The effects of early life trauma on the neurochemistry and behaviour of the adult rat

by

Joachim De Klerk Uys

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DECLARATION

I, the undersigned, hereby declare the the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

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Abstract

Early life trauma leads to behavioural abnormalities later in life. These include mood and anxiety disorders such as depression and posttraumatic stress disorder (PTSD). This association may be due in part to the effects of trauma on brain development. Data from basic and clinical experiments suggest that alterations in the hippocampus may be fundamental to the development of these disorders.

Here we used an animal model of early life trauma to investigate its effects on the behaviour and neurochemistry of the adult rat. Adolescent rats were subjected to time-dependent sensitization stress consisting of a triple stressor (2 hours restraint, 20 min swim stress and exposure to ether vapour) on post-natal day (PND) 28, a single re-stress on PND 35 (20 min swim stress), and a second re-stress in adulthood (PND 60, 20 min swim stress). The rationale was that the frequency of exposure to situational reminders contributes to the maintenance over time of fear-related behavioural disturbances. The effects of trauma on the hypothalamus-pituitary-adrenal-axis, hippocampal and plasma neurotrophin levels, behaviour and phosphoinositide-3 kinase (PI-3 kinase) signaling proteins were initially investigated. In addition, proteomic technologies such as protein arrays and 2D-SDS PAGE combined with liquid chromatography tandem mass spectrometry (LC-MS/MS) were employed to study trauma-induced effects on the hippocampus.

Traumatized animals showed a decrease in glucocorticoid receptors in the dentate gyrus of the hippocampus and an increase in basal corticosterone levels 24 hours after adulthood re-stress. These effects were reversed by pretreatment with the serotonin selective reuptake inhibitor, escitalopram.

A decrease in the neurotrophins, BDNF and NT-3 were evident 8 days, but not 24 hours after adulthood re-stress. This decrease was not accompanied by decreases in plasma neurotrophin or PI-3 kinase, protein kinase B (PKB), phosphatase and tensin homologue (PTEN), phospho-forkhead and phospho-AFX protein levels. In addition, traumatized animals showed increased rearing

in both the elevated plus maze and open field. Proteomic analysis of trauma-induced changes in the hippocampus show increases in Ca^{2+} homeostasis / signaling proteins such as S-100B, phospho-JNK and calcineurin. Apoptotic initiator proteins, including caspase 9, -10 and -12 were increased and there was evidence of cytoskeletal protein dysregulation. Furthermore, cell cycle regulators and energy metabolism proteins were decreased. These effects indicate to a cellular state of cell cycle arrest after increased calcium influx to avoid apoptosis.

Our data suggest that adolescent trauma with adulthood re-stress may affect numerous systems at different levels. These include neuroendocrine-, protein systems and behaviour, and confirmed that a systems biology approach is needed for a better understanding of the neurobiology of mental disorders.

Opsomming

Vroeë lewens trauma kan lei tot patalogiese gedrag in die volwasse lewenstydperk. Hierdie gedrag sluit depressie en posttraumatiese stres steuring (PTSS) in. Die verband tussen trauma en patalogiese gedrag mag moontlik aangespoor word deur die effekte van trauma op breinontwikkeling. Resultate van basiese en kliniese eksperimente het aangetoon dat veranderinge in die hippokampus van kardinale belang is in die ontwikkeling van patalogiese gedrag.

'n Diermodel vir vroeë lewens trauma is gebruik om die effekte op gedrag en neurochemie van die volwasse rot te bestudeer. Adolessente rotte was onderwerp aan tyd-afhanklike sensitisasie stres bestaande uit 'n drievoudige stres paradigma (2 ure immobilisasie, 20 min swem stres en blootstelling aan eter dampe) op lewensdag (LD) 28, 'n tweede swem sessie op LD 35 en 'n laaste swem sessie op LD 60. Die veronderstelling was dat die herhaalde blootstelling aan dieselfde soort stres lei tot die behoud van abnormale vrees gedrag oor 'n lang tydperk. Die effekte van trauma op die HPA-as, neurotrofien vlakke in die hippokampus en plasma, gedrag en PI-3 kinase seintransduksie proteïene was aanvanklik ondersoek. Verder is proteoom tegnologie waaronder proteïen "arrays" en 2D-SDS PAGE wat gekombineer was met kromatografie tandem massa spektrometrie gebruik om effekte van trauma op die hippokampus te bepaal.

Getraumatiseerde diere het minder glukokortikoïed reseptore in die hippokampus en 'n verhoogde basale vlak van kortikosteroon in die plasma 24 uur na volwasse (LD 60) swem stres vertoon. Hierdie effekte was omgekeer deur vooraf behandeling met die selektiewe serotonien heropname inhibitor, escitalopram.

Die neurotrofiene, BDNF en NT-3, was nie 24 uur na volwasse swem stres verlaag nie, maar wel na 8 dae. Hierdie verlaging het nie saam met 'n vermindering in plasma neurotrofiene of PI-3 kinase, PKB, PTEN, fosfo-forkhead en fosfo-AFX proteïenvlakke gepaard gegaan nie. Getraumatiseerde

diere het verder ook meer hiperaktiwiteit in die “open field” en “elevated plus maze” vertoon. Analise van die trauma proteoom het ’n verhoging in kalsium homeostase en seintransduksie proteïene soos S-100B, fosfo-JNK en calcineurin in die hippokampus vertoon. Apoptotiese proteïen vlakke soos kaspase 9, -10 en -12 was verhoog en daar was bewyse van sitoskelet disregulering. Selsiklus reguleerders en energie metaboliese proteïene was verlaag in getraumatiseerde diere. Hierdie effekte dui op ‘n staat van selsiklus stagnering na verhoogde kalsium instroming om apoptose te verhoed.
Ons resultate dui daarop dat adolessente trauma saam met stres in volwassenheid, verskeie sisteme op verskillende vlakke beïnvloed. Dit sluit in neuroendokrien-, proteïen sisteme en gedrag, en bevestig dat ‘n sisteem biologiese benadering nodig is om die neurobiologie van psigiatriese steuringe beter te verstaan.

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Chapter 1
Introduction to the study

1.1 Introduction

Studies have now clearly shown that early life stress leads to a number of behavioural abnormalities often exhibited later in life (Heim and Nemeroff, 2001; Kendler et al., 1992). This is particularly true for mood and anxiety disorders, such as depression and posttraumatic stress disorder (PTSD). Imaging data from clinical experiments suggest that alterations in the hippocampus may be fundamental to the development of these disorders (Duman et al., 1997; Duman et al., 1999; McEwen, 1999). For instance, reduced hippocampal volume has recently been documented in patients diagnosed with depression (Neumeister et al., 2005) and PTSD (Kitayama et al., 2005). Support for the involvement of an aberrant hippocampus in the aetiology of these disorders comes from basic studies where animals, subjected to different stressors (physical, psychosocial, and prenatal stressors) show decreased hippocampal neurogenesis, that may contribute significantly to the observed structural changes in the hippocampus (Czeh et al., 2002; Lemaire et al., 2000).

Early life stress affects hippocampal neurotophin levels (Roceri et al., 2002) and since neurotrophins are growth factors important for the well-being of neurons, changes in their concentrations may be one mechanism by which prolonged stress exerts its deleterious effects. In addition, early adverse events impact negatively on the activity of the hypothalamus-pituitary-adrenal-axis (HPA-axis) (de Kloet et al., 2003), hence one would naturally suspect an interaction between the two systems. However, there is contrasting evidence suggesting both the presence (Smith et al., 1995, Ueyama et al., 1997), as well as an absence (Aloe et al., 1994; Scaccianoce et al., 2000) of an interaction between the HPA-axis and neurotrophins.

1.2 Research objectives

The aim of this study was therefore to characterise the long term effects of early life trauma and adulthood re-stress in an animal model. The study particularly focused on behaviour, HPA-axis activity and hippocampal protein expression. The efficacy of pharmacological treatment in the form of a serotonin selective reuptake inhibitor (SSRI) to reverse the anticipated effects was also investigated. Standard methodologies as well as novel proteomic technologies were employed in this study.

1.3 Research strategy

After carefully studying the literature of animal models of anxiety disorders (chapter 2), it was decided to focus on the time dependent sensitization (TDS) model. Whilst the model has previously been used to study PTSD, we considered it appropriate for early life trauma.

Firstly we addressed the impact of early life stress on HPA-axis activity, concentrating on basal corticosterone release and glucocorticoid receptors in the dentate gyrus of the hippocampus. This receptor location was chosen as it is one of the major sites for neurogenesis (Czeh et al., 2002). Stressed animals were also treated with the SSRI, escitalopram, in order to see whether stress-induced changes were reversible. These experiments are described in Chapter 3.

Subsequently the effects of early life stress on hippocampal neurotrophin expression 24 hours after adulthood re-stress were investigated (Chapter 4). Results from these experiments indicated a dissociation between the HPA-axis and hippocampal neurotrophin levels. Alternatively, our data were interpreted as 24 hours being too early for any significant changes to occur at the level of neurotrophin expression. Following the latter reasoning, behavioural, HPA-axis and hippocampal neurotrophin expression were assessed 8 days post-adulthood re-stress. In addition, plasma neurotrophin were measured in the same animals to determine whether a correlation existed between brain and blood neurotrophin levels. The protein levels of members of the phosphoinositide-3 (PI-3) kinase signaling pathway were also

measured to see whether changes in ligand concentrations were translated to alterations in second messenger signaling (Chapter 5 and 6).
In order to obtain a broader picture of the effects of early life trauma, a multi-systems biological approach was adopted. An overview of current proteomic strategies and their application to human and animal anxiety disorder studies are therefore reviewed in chapter 7, while some of the techniques have been applied in our experiments. Chapter 8 subsequently presents data generated using protein arrays that has been complemented with additional proteomic methods - 2D-SDS PAGE combined with liquid chromatography tandem mass spectrometry (LC-MS/MS). In this study particular attention was given to the effects of early life trauma on ventral hippocampal protein expression, as neurotrophin alterations occurred in this brain area.

1.4 Conclusion

The present study aimed to delineate the effects of early life trauma and adulthood re-stress in the hope of identifying the underlying processes which contribute to the development of psychiatric disorders later in life. We have utilised the TDS / Re-stress model to investigate this phenomenon and obtained interesting results. On a macro level we demonstrated that early life trauma and adult re-stress can lead to behavioural and neuroendocrine abnormalities comparable to that observed in patients suffering from anxiety disorders. These abnormalities were associated with biochemical changes in the hippocampus that included changes in neurotrophin levels and glucocorticoid receptors. Pretreatment with escitalopram was able to reverse the neuroendocrine aberrations. However, proteomic analysis of the ventral hippocampus alone showed that a host of alterations occur within the protein profile following trauma. For instance increases in pro-apoptotic proteins were observed while the levels of regulators of the cell cycle were significantly decreased. Our study therefore underlined the intricacies of behaviour and therefore confirmed that the interplay of complex systems needs to be studied for a better understanding of the neurobiology of mental disorders.

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Chapter 2

Animal models of anxiety disorders.

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Animal Models of Anxiety Disorders

Joachim D.K. Uys [1] , Dan J. Stein [2] , Willie M.U. Daniels [1] , Brian H. Harvey [3*]

[1] Department of Medical Physiology, University of Stellenbosch, Cape Town, South Africa

[2] Department of Psychiatry, University of Stellenbosch, Cape Town, South Africa

[3] Division of Pharmacology, School of Pharmacy, University of Potchefstroom, Potchefstroom, South Africa

* Corresponding author: E-mail: fklbhh@puknet.puk.ac.za

Tel: +27 18 2992238

Fax: +27 18 2992225

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Abstract

Animal models may be useful in investigating the fundamental mechanisms underlying psychiatric disorders, and may contribute to the development of new medications. A computerized literature search (MEDLINE: 1981 – 2003) was used to collect studies on recently developed animal models for anxiety disorders. Particular cognitive-affective processes (e.g. fear conditioning, control of stereotypic movements, social submissiveness, trauma sensitization) may be particularly relevant to understanding specific anxiety disorders. Delineation of the phenomenology and psychobiology of these processes in animals leads to a range of useful models of these conditions. These models demonstrate varying degrees of face, construct, and predictive validity.

Introduction

Animal models can potentially contribute to understanding the mechanisms underlying anxiety disorders, and to screening and developing new medications for their treatment [1; 2]. An initial focus of preclinical work was on the broad construct of “anxiety”, and in particular in addressing the issue of determining whether novel agents had anxiolytic properties [3; 4]. Barbiturates and benzodiazepines, for example, had anxiolytic properties in particular paradigms, and the efficacy of new molecules could be compared to these agents. This approach was, however, problematic insofar as it was not always based on specific cognitive-affective processes relevant to anxiety disorders, and insofar as it was unable to predict the value of various medications (e.g. antidepressants) for human anxiety disorders.

Some models of anxiety have focused on changes associated with acute stress, while others have aimed at understanding the neurobiology of chronic stress (e.g. models of learned helplessness). Models of chronic stress arguably have applicability across a range of psychiatric conditions, including mood [5] and anxiety disorders [6]. Conversely, it has been argued that although these models have provided valuable insights about the neurochemistry and neuroendocrinology of stress responses, they have not led to insights into individual psychiatric disorders. The broad concept of a general stress response, arguably needs to be supplemented by more specific understanding of the mechanisms underlying particular cognitive-affective processes (eg fear conditioning) involved in particular anxiety disorders.

Given the increased evidence that anxiety disorders may have distinctive symptomatology and neurobiology, specific cognitive-affective processes may be particularly relevant to each of the different anxiety disorders. Furthermore, although some of the anxiety disorders seem to be specific to humans, a number of these cognitive-affective processes can be studied in lower animals. This paper discusses models of generalized anxiety disorder (GAD), obsessive compulsive disorder (OCD), panic disorder (PD), social phobia (SP) and posttraumatic stress disorder (PTSD) [7], in each case emphasizing a

cognitive-affective process that may be especially relevant to that disorder. A computerized literature search (MEDLINE: 1981 – 2003) was used to search for studies on recently developed animal models for anxiety disorders.

Generalized anxiety disorder (GAD)

GAD is characterized by excessive and uncontrollable worries about life events. These worries are accompanied by motor tension or hypervigilance [7]. Clinical studies point to dysregulation of monoamine [8] and gamma-aminobutyric acid (GABA) [9] neurotransmitter systems in GAD. Complementing these findings are clinical trials showing that GAD responds reasonably well to benzodiazepines, buspirone, and antidepressants [10].

Development of a behavioral model of GAD is complicated by the fact that core diagnostic criteria for GAD have changed over time [11]. GAD was originally conceptualized as a residual category, for patients' whose anxiety symptoms did not meet criteria for other anxiety disorders. Subsequent DSM definitions of GAD have increasingly focused on "worry", a cognitive symptom that may not have a clear behavioral analogue. Another way of conceptualizing GAD, however, is in terms of heightened activation of innate general avoidance behaviors [6].

General Avoidance Behaviors

A number of animal models based on this principle have been developed. Amongst the best known is the elevated plus- maze (others include the open field test [12] and the stress-induced vocalization model [13]). In the elevated plus maze, a rat or mouse is placed in the center of a maze which has two open and two closed arms, and the animal is allowed to explore freely. The natural fear of open spaces is responsible for the reluctance to explore the maze and fear is measured by the decreased percentage time spent in an open-arm [3]. The elevated plus-maze is sensitive to anxiogenic and anxiolytic agents that act on GABA receptors [3] and to corticotropin releasing factor (CRF) receptor antagonists [14; 15].

This model can be used to investigate a range of potential neurobiological dysfunctions relevant to GAD. For example, mice lacking the 5-HT_{1A} receptor, or 5-HT_{1A} knock-outs (5-HT_{1A} KO), show more anxious behavior in the elevated plus maze [16]. It was also found that diazepam proved anxiolytic in this paradigm, but the effects varied according to the mouse species [17; 18]. Although the gross dysfunction produced by a KO model may differ from the more subtle dysfunctions seen in human psychopathology, KO's have the advantage of being able to study the effects of a single genetic change.

From a phenomenological perspective, it is unclear whether the elevated plus-maze models the core symptom of GAD, i.e. excessive "worry". Furthermore, the elevated plus-maze has a range of methodological problems. These include inter-laboratory differences, and differences between animal strains [19]. Finally, although benzodiazepines reliably reduce anxiety in the elevated-plus maze [3; 19], studies with 5-HT_{1A} agonists and SSRIs have proven inconsistent [20]. This is in contrast to clinical studies that regularly demonstrate serotonergic anxiolytics to be effective in treating GAD [10]. Given these limitations, preclinical work that is intended to address GAD may need to employ a combination of different behavioral models (eg EPM and OFT).

Obsessive-compulsive disorder (OCD)

OCD is characterized by obsessions (recurrent and persistent thoughts), and compulsions (repetitive behaviors or mental acts in response to obsessions) [7]. Clinical studies have emphasized the importance of cortico-striatal circuits in mediating OCD, and have supported the hypothesis that serotonin and dopamine play important roles in mediating the disorder [21; 22]. Selective serotonin reuptake inhibitors (SSRI's) are currently the first-line choice of agent in the treatment of OCD [23] and patients refractory to these agents may respond to augmentation with dopamine blockers [24]. Auto-immuno

processes may play a role in the cortico-striatal dysfunction seen in some OCD patients [25].

Stereotypy is arguably central to OCD, since stereotyped behavior with its repetitive, topographically invariant movements is reminiscent of the compulsions of OCD. Animal models that focus on this phenomenon include the behavioral model of spontaneous stereotypy in deer mice [26; 27; 28; 29], veterinary disorders characterized by stereotypy such as acral lick dermatitis in canines [30], and a number of anatomical/molecular models of repetitive behavior [31; 32; 33]

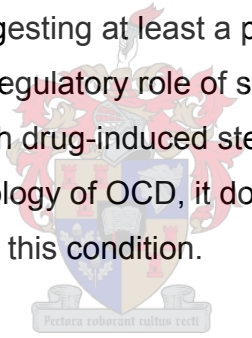
Control of Repetitive Movements

In the rodent model of spontaneous stereotypy, deer mice (*Peromyscus maniculatus bairdii*) express patterns of motor behaviors that are repetitive, excessive and topographically invariant. These behaviors lack any obvious function and purpose [26; 27; 28; 29]. The patterns of motor behavior include patterned running, jumping and backward somersaulting. The DA-agonist, apomorphine, has been found to induce behaviors in non-stereotypic mice that are topographically distinct from behaviors emitted by stereotypic mice. Furthermore, apomorphine only increases two of the three stereotypic behaviors usually emitted by deer mice with no increase in DA receptor sensitivity. Thus, while DA dysfunction may underlie certain aspects of OCD, spontaneous stereotypy is only partially mediated by the dopamine system [27]. The role of the 5-HT system in mediating deermice stereotypy, and its response to administration of different agents, remains to be fully clarified.

Acral lick dermatitis (ALD) is a veterinary disorder characterized by repetitive paw-licking and biting of the extremities in different mammalian species, particularly in large dogs. ALD therefore has some face validity as a model for OCD, insofar as both conditions can arguably be conceptualized as grooming disorders. Furthermore, like OCD, ALD responds more robustly to serotonin reuptake inhibitors than to noradrenergic agents [30]. While the

phenomenology of ALD differs from some subtypes of OCD, stereotypic behaviors in other species are arguably reminiscent of such subtypes (eg rodent hoarding) [34]. Various stereotypies in other animals (eg primates) may also respond to SSRIs [35]. There is a need for additional research to delineate the neurobiological dysfunctions that underlie ALD, and to see whether these are analogous to those responsible for OCD.

Dopamimetic agents such as dex-amphetamine and apomorphine, administered orally or injected into brain regions such as the striatum, have been extensively used to study the neurobiology of stereotypy [36; 37; 38]. For example, rats treated chronically with the dopamine D2/D3 receptor agonist, quinpirole, develop compulsive checking behavior [31; 32] and perform ritual-like behavioral acts at specific places in an open field. Interestingly, the SSRI clomipramine partially attenuates quinpirole-induced compulsive checking [31] suggesting at least a partial role for 5-HT in this behavior, and supporting the regulatory role of striatal 5HT on dopamine-driven behaviors [22]. Although drug-induced stereotypy is only partly reminiscent of the phenomenology of OCD, it does appear to model some aspects of the neurobiology of this condition.



Clinical research suggests that OCD and tics may be mediated by cortico-striatal circuits. One possibility is that such dysfunction involves auto-immune processes [39], and in a preclinical model of this phenomenon, rats were injected with sera from Tourette's syndrome patients with high levels of autoantibodies in the ventrolateral striatum, an area associated with oral stereotypy [37]. Experimental animals exhibited significantly higher oral stereotypy scores (wood chip eating, self-gnawing, biting, licking not associated with grooming, and repetitive paw-to-mouth movements) compared to animals injected with sera from normal subjects or patients with Tourette's syndrome with low autoantibody titers [40]. Although there is evidence that certain immunotherapies may be useful in pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections (PANDAS) [39], parallel preclinical data is not yet available. This model has

phenomenological and neurobiological aspects reminiscent of OCD and deserves further study.

Panic disorder (PD)

PD is characterized by recurrent unexpected panic attacks followed by persistent concern about additional attacks, worry about the implications of the attack or its consequences and a significant change in behavior related to the attacks [7]. A panic attack is normally accompanied by a range of sympathetic symptoms and clinical studies suggest dysregulation of the noradrenergic [41; 42; 43] and serotonergic [44; 45] neurotransmitter systems. Patients with PD respond to various antidepressants including tricyclics (TCA's), monoamine oxidase inhibitors (MAOI's), and SSRIs, and to certain benzodiazepines [46].

Fear conditioning and extinction are processes that may be relevant to the pathogenesis of a number of different anxiety disorders and their treatment [47]. Nevertheless, certain phenomena seen after fear conditioning, such as fear-potentiated startle, are reminiscent of the symptoms of panic disorder (and of the arousal symptoms of PTSD). In this section, we discuss how preclinical studies of fear conditioning may shed light on the neuoroanatomical and molecular basis of panic disorder.

Fear Conditioning

Animal models of conditioned fear examine behaviors that are provoked by stimuli associated with an aversive stimulus, for example an electric foot-shock. Fear conditioning is therefore a form of Pavlovian conditioning where a neutral stimulus is paired with an aversive stimulus (unconditioned stimulus). After a number of pairings the neutral stimulus (conditioned stimulus) elicits fear behaviors without the presence of the aversive stimulus. A major advance has been the delineation of the role of the amygdala and other limbic structures in mediating innate and conditioned fear responses [48; 49; 50].

In animals, stimulation of the amygdala results in behavioral [51] and physiological [52] patterns associated with fear and anxiety, while stimulation of specific target areas of the amygdala produces more selective effects [50]. The extended amygdala may be particularly relevant to anxiety (rather than fear), and the hippocampus plays an important role in contextual fear conditioning. Finally, medial prefrontal cortex (anterior cingulated) plays a crucial role in mediating the extinction of fear conditioned responses. Neurotransmitters that are crucial in this circuitry include the serotonergic, glutamatergic and GABAergic systems.

The fear-potentiated startle response, which consists of fast muscle contraction, especially around the face, neck and shoulders [53], is arguably reminiscent of a panic attack. One of the regions that play an important role in the fear-potentiated startle response is the periaqueductal gray [54]. Stimulation of the dorsal periaqueductal gray (dPAG) elicits fear behaviors and autonomic arousal [55; 56] and lesions of the PAG prevent fear-potentiated startle [57]. Fear-potentiated startle is sensitive to fear-modulating drugs, for example, benzodiazepine agonists [58], 5-HT_{1A} agonists [59] and N-methyl-D-aspartate (NMDA) receptor antagonists [60]. Panicogenic drugs, such as yohimbine and caffeine, lead to an increase in dPAG-induced aversion, while a number of anti-panic drugs, including clonazepam and alprazolam, lead to a decrease [55].

Fear conditioning appears relevant to understanding the development of agrophobia in patients with panic disorder, and arguably provides a conceptual foundation for an integrated approach to extinguishing fear by means of medication or desensitization. Although a fear-potentiated startle model is partly reminiscent of the phenomenology of PTSD, there is arguably overlap with the responses to impending danger also seen in PD [61]. Clinical studies of panic disorder indicate that the neurocircuitry of PD is broader than simply the dPAG. Nevertheless, the dPAG component of this model may have some predictive validity; like PD it responds to clonazepam and alprazolam, imipramine and fluoxetine [62].

There is increasing evidence for a function of amygdala glutamate receptors in fear learning, fear-potentiated startle, and fear extinction [50; 63]. More specifically, amygdala NMDA receptors appear to be involved in the neural changes that support fear learning and also loss of fear that accompanies extinction training [64]. For example, mice lacking a fully functional glutamate NMDA receptor have been found to be less sensitive to stress induced by the elevated plus-maze, light-dark box, and forced swimming tests [65]. Amygdala (±)-α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors also participate in fear learning [66], and glutamate metabotropic group II receptor agonists block fear learning as well as fear-potentiated startle [67].

Augmentation of traditional antidepressants with NMDA antagonists in various animal models of stress and depression [68], and their ability to re-establish anti-stress efficacy after antidepressant withdrawal [69], hints at the potential of future treatment strategies with glutamatergic agents [70; 71]. Nevertheless, there is to date limited evidence for the role of glutamate in PD and for the efficacy of NMDA-antagonists in the treatment of this disorder.

Social Phobia

Social phobia is characterized by excessive fear of social and performance situations. Patients may experience panic attacks in such situations, and tend to avoid them [7]. Patients with social phobia respond to SSRI's [10], suggesting an involvement of the serotonin system, although there is also evidence from clinical studies of dopaminergic involvement [72].

Some authors have suggested that social phobia is a uniquely human condition. Nevertheless, social submissiveness is seen in lower animals, and may constitute a cognitive-affective process that proves useful for studying the neurobiology of social phobia.

Social Submissiveness

Social submissiveness has been a particular focus of attention in studies of non-human primates. For example, social status and degree of social affiliation are associated with altered HPA-axis function among free-ranging wild baboons [73]. Socially subordinate baboons exhibit hypercortisolism and resistance to feedback inhibition after dexamethasone treatment. Nevertheless, it is unclear that social phobia is characterized by HPA-axis dysfunction [74].

Lower social status in monkeys is, however, associated with lower dopamine D2 striatal receptor density [75], a finding that is consistent with clinical research on social phobia [76]. Furthermore, social submissiveness in non-human primates decreases in response to administration of SSRIs [77]. These kind of data lend support to the thesis that social phobia can be conceptualized in terms of an appeasement display [78].

Posttraumatic stress disorder (PTSD)

PTSD develops after an individual has experienced or witnessed a life-threatening traumatic event. The symptoms include re-experiencing the traumatic event (for example flashbacks and nightmares), generalized arousal, and avoidance of stimuli associated with the trauma [7]. Clinical studies have implicated the amygdala and hippocampus [79], and have demonstrated enhanced negative feedback of the HPA-axis [80] and dysregulation of catecholamine neurotransmitter systems [81]. Patients diagnosed with PTSD respond to a range of medications, including TCAs, MAOIs, and SSRI's [10].

Animal models of PTSD have utilised intense stressors, aversive challenges, and situational reminders of a traumatic stress, in an attempt to model long-term effects on behavioral, autonomic, and hormonal responses seen in humans with PTSD. Examples include electric shock [82], stress–restress or time-dependent sensitization (TDS) [83], underwater trauma [84], and exposure of animals to a predator [85; 86]. Models of early developmental

trauma [87; 88] may also be relevant to understanding PTSD. We will focus here on the process of time-dependent sensitization.

Time-dependent sensitization (TDS)

The behavioral model of stress-restress or time dependent sensitization (TDS) has been proposed as a useful model for PTSD [89]. In this model, animals are exposed to single session of prolonged stress (eg, 2hr restraint followed by a 20 minute forced swim, followed by exposure to ether or halothane vapors). The animals are allowed to recover for a week, whereafter they are subjected to a brief restress on day 7 (30 minutes of restrain stress or 20 minutes swim stress). The rationale being that the frequency of exposure to situational reminders contributes to the maintenance over time of fear-related behavioural disturbances.

The model has proved valuable for studying HPA abnormalities relevant to PTSD [83]. Animals subjected to TDS display the enhanced sensitivity to negative glucocorticoid feedback that is characteristic of PTSD. In addition stress-restress evokes significant spatial memory deficits together with lowered plasma corticosterone, again consistent with clinical findings [90]. Stress-restress leads to changes in hippocampal $5HT_{1A}$ and pre-frontal cortex $5HT_{2A}$ receptors [90], brain areas that are intimately involved in memory and stress responsiveness.

From a phenomenological and biological perspective the TDS model emphasizes the role of prior trauma in predicting subsequent dysfunction, allows for the study of bidirectional expression of symptoms (enhanced or reduced responsiveness to environmental stimuli), and provides credible intra-subject variation [89]. Interestingly, TDS-induced stress effects on spatial memory are attenuated by both fluoxetine, and by the steroid synthesis inhibitor, ketoconazole [91]. Moreover, in line with the increasing evidence for an involvement of glutamatergic mechanisms in the pathology and pharmacology of stress and anxiety [70; 71], it is of interest that stress-restress evokes a significant increase in hippocampal nitric oxide synthase

activity, together with marked changes in hippocampal NMDA receptors [92]. The efficacy of other anti-PTSD agents in the TDS model still remains to be proven.

With advances in genomics, it will be increasingly possible to explore the specific genetic basis of individual differences in processes such as TDS, and susceptibility to PTSD [93]. A genetic animal model of congenital learned helplessness (cLH), for example, has been used to explore the role of genetic predisposition in PTSD [94]. The first cLH breeding line was selected by subjecting out-bred Sprague-Dawley rats to random electric foot-shocks. Twenty-four hours later the animals were tested in a shock-escape paradigm where foot-shock could be eliminated by a single bar press. Failure to eliminate the shock in 20 seconds counted as a failed trail. Rats scoring 11-15 failed trials in a 15-trial session were labeled cLH. Animals from the 33rd breeding line of cLH animals were monitored for changes in pain tolerance, spatial memory and hypothalamic-pituitary-adrenal function after re-exposure to intermittent stress. Stress-induced analgesia was significantly increased in cLH animals. cLH animals also exhibited significant deficits in spatial memory as measured by the Morris water maze. In addition cLH animals exhibited HPA-hyporesponsitivity to major stressors possibly due to enhanced negative feedback sensitivity [94]. A decrease in pain sensitivity [95], impairment in memory [96], and enhanced negative feedback sensitivity [80] are all features of PTSD. Further work is needed to determine whether such animals are more at risk for developing adverse consequences after TDS, and whether pharmacotherapy is effective in reversing dysfunctions in cLH animals.

Conclusions

There is a growing understanding of the phenomenology and psychobiology of specific cognitive-affective processes (eg fear conditioning, social submissiveness, trauma sensitization) that may be relevant to the anxiety disorders. While some of these processes are relevant to several different anxiety disorders, others (eg control of stereotypic behaviors) are particular

pertinent to specific conditions. Although cognitive-affective processes in humans may have unique attributes, it is possible to study such processes in lower animals.

Indeed, such work has led to a number of animal models of anxiety that demonstrate varying degrees of face, construct, and predictive validity (summarized in Table 1). These models have broadened our understanding of the neuroanatomy and neurochemistry of anxiety disorders, highlighting regions such as the amygdala and hippocampus, and systems such as serotonergic and glutamatergic circuitry. In the future, as new technologies become available to explore the precise molecular and genetic bases of cognitive-affective processes relevant to anxiety disorders, we can expect further progress. Ultimately, such work may lead to the development of novel treatment approaches.

Table 1: Cognitive-affective processes relevant to the molecular and anatomical basis of anxiety disorders, as modelled by various pre-clinical models of anxiety

Disorder	**Cognitive Affective Process**	**Paradigm**	**Anatomy**	**Molecular**
GAD	General avoidance behaviors	Elevated plus-maze	Poorly defined	5-HT, GABA
OCD	Control repetitive movements	Spontaneous stereotypy (deermice), ALD (canines), Drug-induced stereotypy	Cortico-striatal circuits	5-HT, DA, auto-immune
PD	Fear conditioning	Fear potentiated startle	Amygdala, hippocampus, medial prefrontal cortex, dPAG	5-HT, glutamate, GABA
SP	Social submission	Primate hierarchy	Amygdala, cortico-striatal circuitry	5-HT, D_2
PTSD	Time-dependent sensitization	Rodent TDS model	Hippocampus, prefrontal cortex	HPA-axis, 5-HT,

Refer to text for abbreviations and further description

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Chapter 3

Early life stress decreases glucocorticoid receptors in rat dentate gyrus upon adult re-stress: reversal by escitalopram.

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Early life trauma decreases glucocorticoid receptors in rat dentate gyrus upon adult re-stress: reversal by escitalopram

Joachim D.K. Uys [1], Christo J.F. Muller [2], Lelanie Marais [1], Brian H. Harvey [3], Dan J. Stein [4], Willie M.U. Daniels [1]

1) MRC Research Unit for Anxiety and Stress-related Disorders, Department of Medical Physiology, University of Stellenbosch, Tygerberg, South Africa
2) Department of Anatomy and Histology, University of Stellenbosch, Tygerberg, South Africa
3) Department of Pharmacology, University of the North-West, Potchefstroom, South Africa
4) Department of Psychiatry, University of Cape Town, South Africa

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* Corresponding author:
Joachim D.K. Uys
MRC Research Unit for Anxiety and Stress-related Disorders
Department of Medical Physiology
University of Stellenbosch
Tygerberg
7505
South Africa
Tel: +27 21 938 9531
Fax: +27 21 938 9476
Email: jduys@sun.ac.za

Abstract

Early exposure to adverse experiences may lead to specific changes in hippocampal glucocorticoid (GC) function resulting in abnormalities within the hypothalamic-adrenal (HPA) axis. Given interactions between the neuroendocrine and central serotonergic systems, we hypothesized that exposure to early trauma would lead to abnormal HPA axis activity that would be normalised by pretreatment with a serotonin selective reuptake inhibitor (SSRI). HPA axis function was assessed by determining basal corticosterone levels and hippocampal glucocorticoid receptor immunoreactivity. Rats were subjected to a triple stressor on post-natal day (PND) 28, and again to a single swim re-stress session on PND 35 and PND 60. On PND 61 i.e. 24 hours after the last re-stress, tail-vein blood was collected for serum corticosterone determinations and hippocampal tissue was collected for immunohistochemistry of GC receptors. Escitalopram (5mg/kg) or saline vehicle was administered from PND 47 – PND 60 via osmotic mini-pumps. Animals exposed to early life trauma showed an increase in basal corticosterone levels, and a significant decrease in the ratio of GC receptor positive cells to total cells in the hilus, granule cell layer and the dentate gyrus. Both the increase in basal corticosterone and decrease in GC receptor imunoreactivity were reversed by escitalopram pretreatment. These data confirm alterations in HPA axis function that may stem from decreases in GC receptor levels, in response to early adverse experiences, and demonstrate that these alterations are reversed by SSRI pretreatment.

Keywords:
trauma, hippocampus, corticosterone, SSRI, immunohistochemistry

It has been suggested that the association between early life trauma and later psychopathology (Heim and Nemeroff, 2001; Kendler et al., 1992) is mediated by specific effects on hippocampal plasticity (Kaufman et al., 2000). Preclinical and clinical studies provide evidence for trauma-induced atrophy and loss of hippocampal neurons (Duman et al., 1997, 1999; McEwen et al., 1999). For example, brain-imaging studies indicate that hippocampal volume is reduced in disorders associated with early trauma, such as depression and posttraumatic stress disorder (Drevets, 2003; Grossman et al., 2002; Hull, 2002). Given the stress-induced down-regulation of glucocorticoid (GC) receptors and glucocorticoid receptor mRNA (Kim et al., 1999; Ladd et al., 2004; Workel et al., 2001), it has been proposed that changes in hippocampal structure may in fact be mediated by glucocorticoids. (Czeh et al., 2002; Lemaire et al., 2000; McEwen, 2000).

Antidepressants also have actions on glucocortioid (GC) receptor expression. For example, in cultured rat primary hippocampal cultures, citalopram (Hery et al., 2000) increased GC receptor binding sites. However some in vivo studies showed changes in GC receptor expression after treatment with a selective serotonin re-uptake inhibitor (SSRI) (Brady et al., 1992; Yau et al., 2002), while others found no differences (Bjartmar et al., 2000; Budziszewska et al., 1994). Interestingly, chronic in vivo treatment with the antidepressants, fluoxetine and reboxetine, increased neurogenesis in the adult hippocampus of normal animals (Malberg et al., 2000). On the other hand, stress-induced changes in hippocampal neurogenesis were prevented by prior treatment with the antidepressant tianeptine (Czeh et al., 2001).These studies therefore indicate that the effects of antidepressants on hippocampal biology remains unclear.

Escitalopram, the S-enantiomer of the SSRI citalopram, has proved to be effective in preclinical models of depression and anxiety, and was more potent than its racemate in inhibiting 5-HT transporter functions, 5-HT neuronal firing in the dorsal raphe nucleus and in potentiating 5-hydroxytryptophan-induced

behaviours (Sanchez et al., 2003). The present study therefore evaluated the effects of early life trauma combined with adulthood re-stress on HPA axis activity, GC expression in the hippocampus and examined the capacity of escitalopram to reverse the trauma-induced changes. We hypothesized that HPA axis function would be altered, GC receptor immunoreactivity in the hippocampus would be reduced in rats subjected to early life trauma, and that 14 days of pretreatment with escitalopram would reverse these effects.

Experimental Procedures

Animals

The animal studies were approved by the ethics committee of the University of Stellenbosch. All experiments conformed to the Helsinki convention for the use and care of animals. Care was taken to minimize the number of animals and their suffering. Male Sprague-Dawley rats were used. Animals were housed in pairs under standard laboratory conditions (12/12 hours light/dark cycle, food and water ad libitum) in the central animal research facility of the University of Stellenbosch.

Animals were divided into four experimental groups: control + vehicle, control + escitalopram, trauma + vehicle and trauma + escitalopram. A total of 54 animals were used, 21 of these animals were randomly selected for immunohistochemistry.

Trauma procedure

The trauma procedure is based on the TDS model where animals are subjected to a single exposure of sequential stressors of escalating severity followed by a single exposure to one of the stressors, 7 days later (Liberzon et al., 1997; Yehuda and Antelman, 1993; Harvey et al, 2003). The rationale is that the frequency of exposure to situational reminders contributes to the maintenance over time of fear-related behavioural and neurochemical disturbances. Young rats were subjected to a triple stressor on PND 28. The triple stressor consisted of being firstly placed in a plexiglass restrainer for 2hrs with the tail-gate adjusted to keep the rat well contained without impairing circulation to the limbs; secondly the rats were placed individually in 18 cm of

ambient water (25°C) in a perspex swim tank and forced to swim for 20 minutes; and finally after gently drying the rat with a soft towel and 30 minutes recovery, each rat was then exposed to ether vapours until loss of consciousness. The animals were then removed and placed in their home cages.
Traumatized animals were exposed to situational reminders by subjecting them to swim stress on PND 35 and again during adulthood on PND 60.

Escitalopram treatment

Escitalopram (5mg/kg, kindly donated by H. Lundbeck A/S, Copenhagen, Denmark) or saline vehicle was administered for 14 days (PND 47 – PND 60) via osmotic mini-pumps (ALZET, Cupertino, CA, USA. model 2ML2), implanted subcutaneously on PND 46. Animals were anesthetized with Equithesin mixture (pentobarbitone 6% and 0.25M chloral hydrate) and placed on a heating pad during surgery to prevent hypothermia. After implantation two animals were placed per cage with a clear, perforated Perspex divider between them, creating two compartments. This allowed the animals to exchange olfactory, visual and auditory cues without inducing total isolation stress. The divider prevented the animals from physically reaching each other and possibly dislodging the sutures of the mini-pump or injures its co-inhabitant. After surgery the cage was placed under a heating lamp to again prevent hypothermia, until the animals were fully recovered.

Corticosterone determination

On PND 61 i.e. 24 hours after the last swim stress, serum and hippocampal tissue samples were collected for corticosterone determination and GC immunihistochemistry respectively. Blood was collected under Equithesin anesthesia via tail snip on PND 61 in prechilled 1.5ml Eppendorf tubes. All blood samples were collected between 9.00 and 11.00 am. After collection blood samples were centrifuged (5 minutes, 14000 rpm, 4^{o}C) and serum samples were stored in liquid nitrogen until measurement. Serum corticosterone (CORT) was measured using commercially available RIA kits (IBL, Germany).

Immunohistochemistry

While the animals were deeply anesthetized, they were perfused transcardially with 100 ml ice cold saline followed by 500 ml fixative containing 4% paraformaldehyde in 0.1 mol/L phosphate-buffered saline (PBS; pH 7.4) on PND 61. After perfusion the brains were removed, stored in fixative at 4^{o}C overnight and transferred into 30% sucrose at 4^{o}C for 24 hours and thereafter frozen at –80^{0}C until sectioning.

A cryostat was used to collect serial coronal sections (30 μm) through the hippocampus (plates 26 – 40, Paxinos and Watson, 1986). Every 10th section was selected for immunohistochemistry. All sections were mounted and coded before processing to ensure objectivity.

For glucocorticoid receptor (GR) peroxidase staining, sections were heated in a microwave for 5 minutes (100W) in sodium citrate buffer, cooled down for 5 minutes, heated again (100W) for 10 minutes, cooled down for 20 minutes and finally rinsed in distilled water. This was followed by extensive washing in PBS, preincubated for 10 min in 3% H_2O_2 (to block endogenous peroxidases). Then the sections were preincubated for 1 h with PBS containing 0.5% Tween-20 and 1% bovine serum albumin (BSA), and subsequently incubated for 24 h at 4^{0}C in rabbit polylonal anti-GR antibody (1:50; Santa Cruz) diluted in PBS containing 0.5% Tween-20 and 1% BSA. The sections were then incubated for 30 min with a biotin-labeled horse anti-rabbit/mouse antibody (Dako LSAB 2 Universal kit) followed by amplification with an avidin-biotin complex (Dako LSAB 2 Universal kit) and visualized with DAB (Dako LSAB 2 Universal kit).

Slides were counterstained with hematoxylin, dehydrated, cleared and mounted with a coverslip under permanent mounting media (Dako).

For GR immunofluorescence, sections were heated in a microwave for 5 minutes (100W) in sodium citrate buffer, cooled down for 5 minutes, heated again (100W) for 10 minutes, cooled down for 20 minutes and rinsed in distilled water. This was followed by extensive washing in PBS, preincubation of 1 hour in PBS containing 0.5% Tween-20 and 1% BSA. Sections were then incubated for 24 h at 4 ^{0}C with rabbit anti-GR (Santa Cruz, 1:50) and mouse

anti-NeuN (Chemicon, 1:100) diluted together in PBS containing 0.5% Tween-20 and 1% BSA.
The sections were rinsed in PBS and incubated for 1 h in the dark at room temperature with goat anti-mouse Alexa 594 and goat anti-rabbit Alexa 488 (both highly cross absorbed, 1:200, Molecular Probes) diluted together in PBS containing 0.5% Tween-20 and 1% goat serum. Slides were rinsed, dried and covered under Prolong Gold anti-fade reagent (Molecular Probes).

Glucocorticoid receptor quantification

Cells were examined under 40 x magnification with a Zeiss microscope and Zeiss KS300 image analyser and processed with Zeiss 3.0 software. Five sequential coded slices per animal were examined for GR positive cells in the right dentate gyrus (i.e. slide number 31, 41, 51, 61, 71). All GR positive cells within the hilus were counted regardless of size and shape. In addition, all GR positive cells in two randomly chosen areas in the granule cell layer were counted regardless of size and shape (see figure 1 for areas selected in each section).
GR positive cells within the subgranular zone that were within two cell body widths of the granule cell layer were considered part of the granule cell layer. Total number of GR positive cells in the dentate gyrus (granule cell layer + hilus) and hilus or granule cell layer alone were expressed as a ratio of the number of positive cells to total cells (stained with hematoxylin) in a given area.

Glucocorticoid receptor immunofluorescence

In each animal co-localization of GR positive cells with neurons were examined using a Nikon fluorescence microscope under 20 x magnification.

Statistical analysis

Statistical analyses were done by the Centre for Statistical Consultation at the University of Stellenbosch. The data was analyzed with a one way ANOVA with SPSS version 11.0. When significant differences were obtained, post-hoc comparisons were performed using the LSD test. Data was considered significant if $p \leq 0.05$.

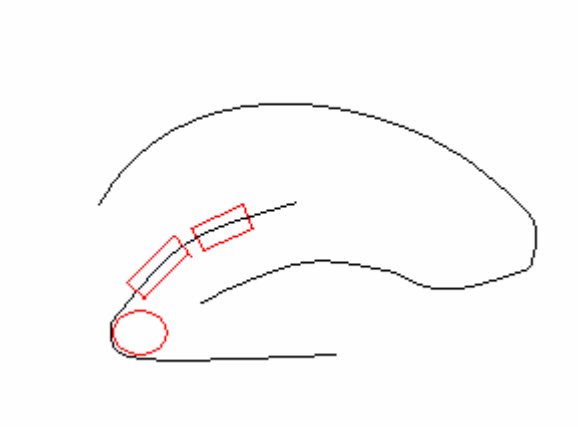


Figure 1: Areas (one in the hilus and two in the granule cell layer) selected in the right dentate gyrus for glucocorticoid receptor immunoreactivity of traumatized and control animals.

Results

Corticosterone levels

There was an overall significant effect in basal corticosterone levels between the groups [$F(3, 50) = 5.553$, $p \leq 0.01$] (figure 2). Traumatized animals treated with escitalopram did not differ significantly from saline controls ($p = 0.38$), but showed a decrease in corticosterone compared to traumatized animals treated with saline ($p \leq 0.01$). Furthermore, traumatized animals treated with saline displayed an increase in basal corticosterone levels compared to saline treated controls ($p \leq 0.05$). Control animals treated with escitalopram did not differ significantly from saline controls ($p = 0.15$).

Immunohistochemistry

GC receptor staining showed significant changes in GC receptor density in the granule cell layer. Animals that were exposed to trauma (figure 3a) exhibited diminished GC receptor positivity compared to controls (figure 3b). Traumatized animals treated with escitalopram (figure 3c) showed similar GC receptor positivity as saline controls (figure 3b). These observations were confirmed by our quantification methods (see below).

Glucocorticoid receptor quantification

There were overall significant effects between groups. Similar results were obtained for the hilus [$F(3,17) = 4.647$, $p \leq 0.05$], granule cell layer [$F(3,17) = 5.778$, $p \leq 0.01$] and the dentate gyrus [$F(3,17) = 5.842$, $p \leq 0.01$]. Traumatized animals treated with saline showed a decrease in the ratio of GC receptor positive cells/total cells compared to saline controls in the hilus ($p \leq 0.01$), the granule cell layer ($p \leq 0.01$) and the dentate gyrus ($p \leq 0.01$). These decreases were reversed by escitalopram pretreatment in all three areas and did not differ from saline controls; hilus ($p = 0.54$), granule cell layer ($p = 0.37$) and dentate gyrus ($p = 0.38$) [figures 4a, 4b and 4c]. Interestingly, control animals treated with escitalopram also displayed significant decreases in the ratio GC receptor positive cells/total cells compared to saline controls in the

hilus ($p \leq 0.05$), granule cell layer ($p \leq 0.01$) and dentate gyrus ($p \leq 0.01$) and did not differ from traumatized animals treated with saline; hilus ($p = 0.61$), granule cell layer ($p = 0.96$) and dentate gyrus ($p = 0.79$).

Glucocorticoid receptor immunofluorescence

In our investigation as to the position of GC receptors, we found a high degree of co-localization of GC-receptor positive cells with neurons (figures 5).

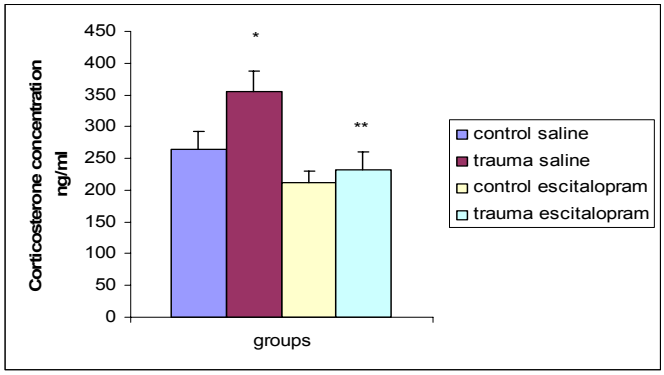


Figure 2: Serum corticosterone levels of control and traumatized animals with or without escitalopram treatment. Values are expressed as mean ± SEM in ng/ml. Traumatized animals were subjected to a triple stressor on PND 28 (restraint stress, swim stress and ether inhalation), and to swim stress alone on PND 35 and PND 60. Blood was collected form the tail vein on PND 61. Escitalopram treatment was administered via osmotic pumps from PND 47-61 at a dose of 5 mg/kg. * $p \leq 0.05$, control saline vs. trauma saline. ** $p \leq 0.01$, trauma saline vs. trauma escitalopram

Figure 3a:

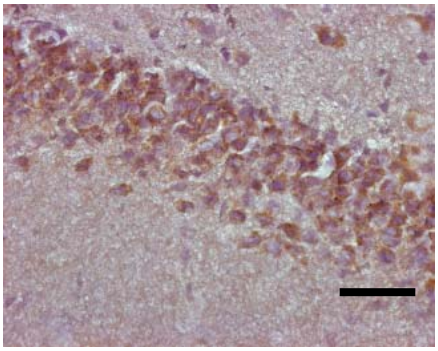


Figure 3b:

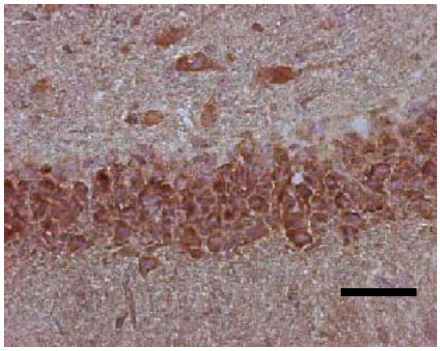


Figure 3c:

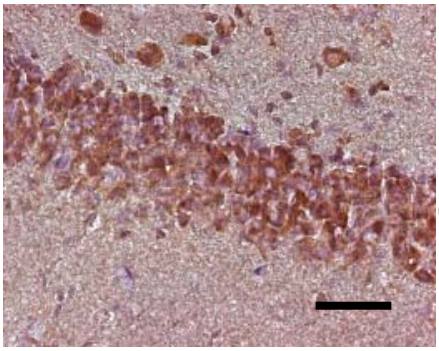
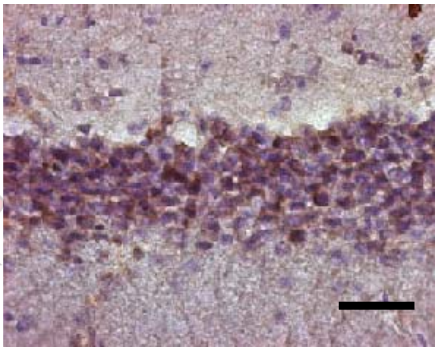


Figure 3d:



Pectora roborant cultus recti

Figure 3: Representative photomicrographs of GC receptor immunoreactivity in the granule cell layer of traumatized (a), control (b), traumatized + escitalopram-treated (c) and control + escitalopram-treated (d) animals. Photomicrographs show traumatized animals having decreased GC receptor immunoreactivity compared to controls (a vs b), while traumatized + escitalopram-treated animals show no differences compared to controls (c vs b).

(Scale bar in above figures 1cm = 30 μm)

Figure 4a:

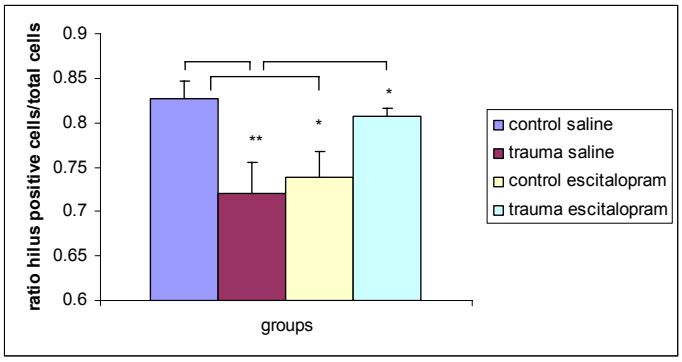


Figure 4b:

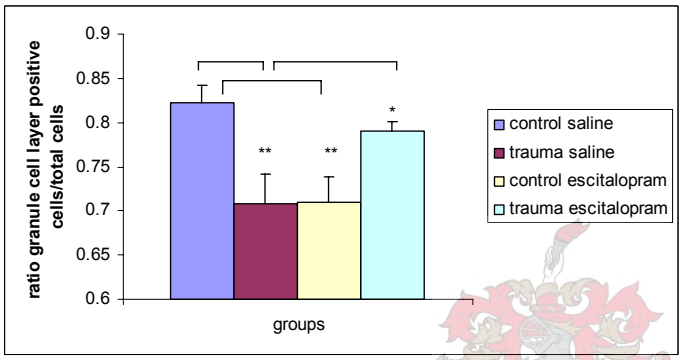


Figure 4c:

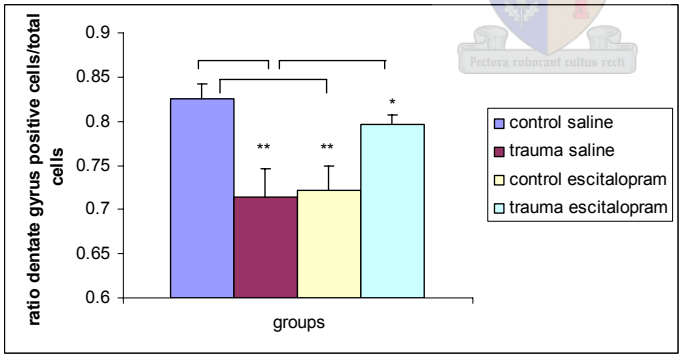


Figure 4: Histograms depicting the ratio of glucocorticoid receptor positive cells to total cells in the hilus (a), granule cell layer (b) and dentate gyrus (c) of traumatized animals. *p ≤ 0.05, ** p ≤ 0.01.

Figure 5a:

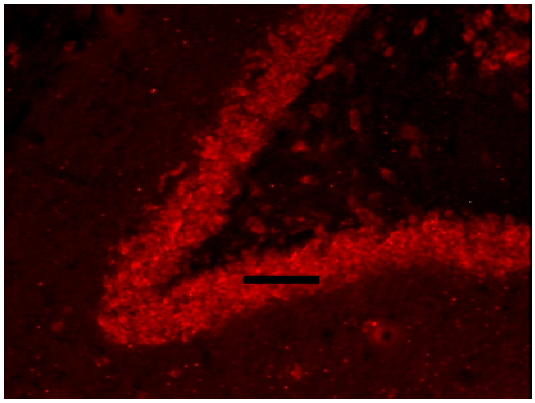


Figure 5b:

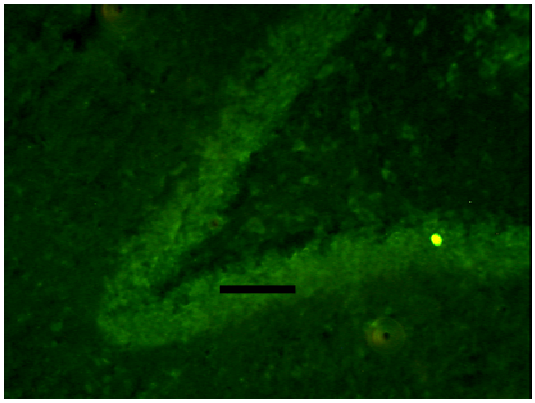


Figure 5c:

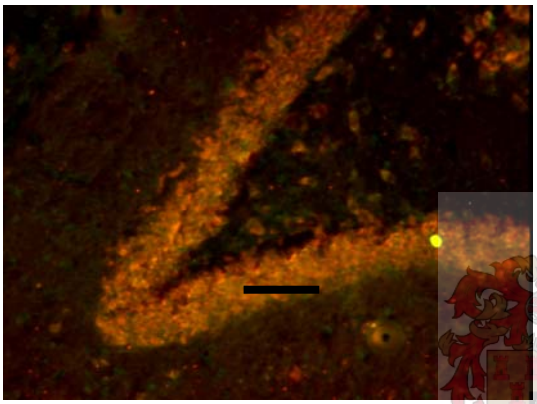


Figure 5: Photomicrographs showing anti-NeuN labeled cells (a) in the dentate gyrus demonstrating immunoreactivity in the hilus and granule cell layer throughout, anti-GC receptor labeled cells (b) showing GC reactive cells in the dentate gyrus, and anti-NeuN and anti-GC receptor labeled cells merged (c) to demonstrate co-localization of neurons and GC receptor immunoreactivity in the dentate gyrus
(Scale bar 1cm = 50 μm)

Discussion

The main findings of the present study were that early life trauma 1) alters HPA axis function, 2) leads to changes in GC receptor immunohistochemistry in the hippocampus as reflected by decreased GC receptor immunoreactivity in the hilus, granule cell layer and dentate gyrus, and 3) that these changes could be reversed by pretreatment with the SSRI, escitalopram.

The reduction in glucocorticoid receptor immunoreactivity as observed in animals that were subjected to early life trauma, is in accordance with studies demonstrating early life stress to decrease glucocorticoid receptor mRNA in adulthood (Kim et al., 1999; Ladd et al., 2004; Workel et al., 2001). A decrease in hippocampal glucocorticoid receptors implies decreased negative feedback inhibition of the HPA axis, resulting in higher levels of circulating corticosterone (Vasquez et al., 1996; Yehuda, 2001). This is in agreement with the corticosterone results found in this study. It is important to note that no significant differences in total cell count per area between the groups (data not shown), and therefore the differences in immunoreactivity were considered related to changes in GC receptor expression and not to loss in cell number per se.

Pectora roborant cultus recti

In the early life traumatized animals the decrease in glucocorticoid receptor immunoreactivity was effectively blocked by escitalopram (14 days, 5mg/kg/d). That these beneficial effects of the drug strongly involve the serotonergic system is very likely (Sanchez et al., 2004), given recent evidence that trauma-induced neuroreceptor and memory changes can be modified by serotonin enhancing and depleting drugs (Harvey et al, 2004). In support, long-term exposure to high circulating levels of glucocorticoids following repeated trauma, attenuate 5HT levels in the hippocampus, thereby effectively removing 5-HT_{1A} receptor-mediated inhibition of the hippocampus (Karten et al, 1999). The fact that chronic administration of escitalopram reversed a putative hyposerotonergic state induced by the trauma, and that both the elevated basal corticosterone levels and the reduction in GC immunoreactivity were normalized by this treatment, reaffirms a causal role of

altered 5-HT in this response. Such a mechanism is plausible as increased serotonergic activity following SSRI administration has been shown to restore HPA axis malfunction and glucocorticoid release via 5-HT_{1A} receptors (Cassano and D'Mello, 2001).

While it has been shown that escitalopram is effective in reducing ultrasonic vocalizations in maternally separated mouse pups, indicating anxiolytic effects (Fish et al., 2004), this is the first study to demonstrate escitalopram to prevent GC receptor expression in the hippocampus of an animal model of early life trauma. Another interesting finding is that control animals treated with escitalopram also showed reductions in GC receptor immunoreactivity in the hilus, granule cell layer and dentate gyrus, similar to traumatized controls. To our knowledge, there is no data on the effects of chronic escitalopram treatment on GC receptor expression in the hippocampus of normal control subjects. However some work has been performed with the racemate, citalopram. For instance animals treated with citalopram (20mg/kg/d) for 14 days, showed no differences in GC receptor mRNA expression (Seckl and Fink, 1992) and 21 day treatment did not modify binding parameters of GC receptors in the rat hippocampus (Budziszewska et al., 1994). It has however, been reported that citalopram induced an increase in GR binding sites in cultured rat primary hippocampal cultures (Hery et al., 2000).

There is limited data available on chronic escitalopram treatment in healthy human subjects. Acute administration of escitalopram (10mg) to healthy human subjects resulted in an increase in salivary and plasma cortisol levels (Nadeem et al., 2004). Chronic treatment of healthy human subjects with another SSRI, fluoxetine, resulted in no changes in mood (Gelfin et al., 1998) or cerebral blood flow (Bonne et al., 1999). These observations reveal that the effects of both acute and chronic antidepressants in healthy subjects remain controversial.

In summary, early life trauma caused an elevation in basal corticosterone secretion that was associated with decreased glucocorticoid receptor immunoreactivity in the hilus, granule cell layer and dentate gyrus of the

hippocampus. These changes were prevented by escitalopram pretreatment, indicating interactions between the neuroendocrine and the serotonergic system. Future work is needed to delineate the exact nature of this interaction.

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Chapter 4

Early trauma decreases glucocorticoid receptor immunoreactivity in the rat dentate gyrus following adulthood re-stress and is reversed by escitalopram pretreatment

European Neuropsychopharmacology 2005, 15:suppl3:s364 [abstract]

Early trauma decreases glucocorticoid receptor immunoreactivity in the rat dentate gyrus following adulthood re-stress and is reversed by escitalopram pretreatment

Joachim D.K. Uys [1], Christo J.F. Muller [2], Lelanie Marais [1], Brian H. Harvey [3], Dan J. Stein [4], Willie M.U. Daniels [1]

1) MRC Research Unit for Anxiety and Stress-related Disorders, Department of Medical Physiology, University of Stellenbosch, Tygerberg, South Africa
2) Department of Anatomy and Histology, University of Stellenbosch, Tygerberg, South Africa
3) Department of Pharmacology, University of the North-West, Potchefstroom, South Africa
4) Department of Psychiatry, University of Cape Town, South Africa

* Corresponding author:
Joachim D.K. Uys
MRC Research Unit for Anxiety and Stress-related Disorders
Department of Medical Physiology
University of Stellenbosch
Tygerberg
7505
South Africa
Tel: +27 21 938 9531
Fax: +27 21 938 9476
Email: jduys@sun.ac.za

Purpose of study: Early exposure to traumatic experiences may lead to specific changes in hippocampal glucocorticoid (GC) function, which is especially observable during adult re-stress. Given interactions between GC receptors, neurotrophins, and the serotonin system, we hypothesized that after exposure to early trauma, neurotrophin levels would decrease in response to re-stress, and that pre-treatment with escitalopram, a selective serotonin reuptake inhibitor (SSRI) antidepressant, would normalize GC receptor immunoreactivity in response to re-stress.

Methods: Animals were subjected to a triple stressor (2 hours restraint, 20 min swim stress and exposure to ether vapour) on post-natal day (PND) 28, a single re-stress on PND 35 (i.e. time dependent sensitization (TDS) model), and a second re-stress in adulthood (PND 60). Hippocampal tissue was collected 24 hours post adulthood re-stress (second re-stress) for immunohistochemistry of GC receptors and analysis of brain derived neurotrophic factor (BNDF), nerve growth factor (NGF) and neurotrophin 3 (NT-3) levels by ELISA. Escitalopram (5mg/kg) and saline vehicle was administered from PND 47 - PND 60 via osmotic mini-pumps.

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Results: Animals exposed to early life trauma showed no differences in NGF, BDNF or NT-3 (see figure 1a, 1b and 1c) in dorsal or ventral hippocampus compared to controls 24 hours post adulthood re-stress (second re-stress). However, there was a significant overall effect between groups at this time in glucocorticoid (GC) receptor immunoreactivity in the hilus [$F(3,21) = 4.647$, $p = .015$], granule cell layer [$F(3,21) = 5.778$, $p = .007$] and dentate gyrus [$F(3,21) = 5.842$, $p = .006$]; TDS animals showed a decrease in GC receptor positivity compared to controls in the hilus ($p = .007$), granule cell layer ($p = .004$) and dentate gyrus ($p = 003$). TDS animals treated with escitalopram showed an increase in GC receptor positivity compared to TDS animals treated with saline in the hilus ($p = .018$), granule cell layer ($p = .023$) and dentate gyrus ($p = .016$), to the extent that measures were similar to those seen in non-traumatized controls.

Conclusion: These data are consistent with previous work showing decreases in hippocampal GC receptor levels in response to early trauma, and with work showing that SSRIs act to increase GC receptor levels. The data confirm our hypothesis that decreases in GC receptor immunoreactivity in the hippocampus following early traumatic experience are reversed by escitalopram pretreatment. Additional delineation of the interactions between signal transduction proteins of GC receptors, neurotrophins and the serotonergic system is required.

Figure 1a:

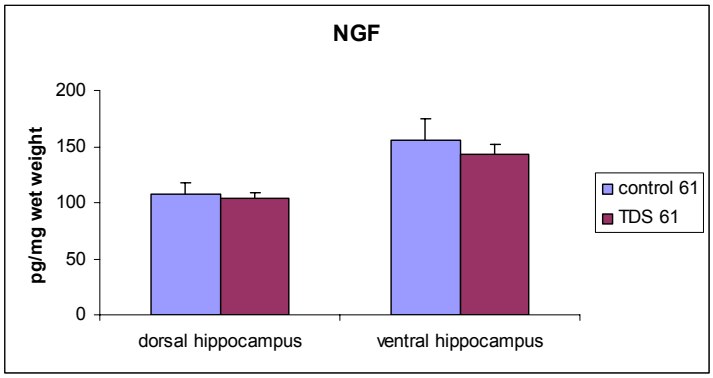

Figure 1b:

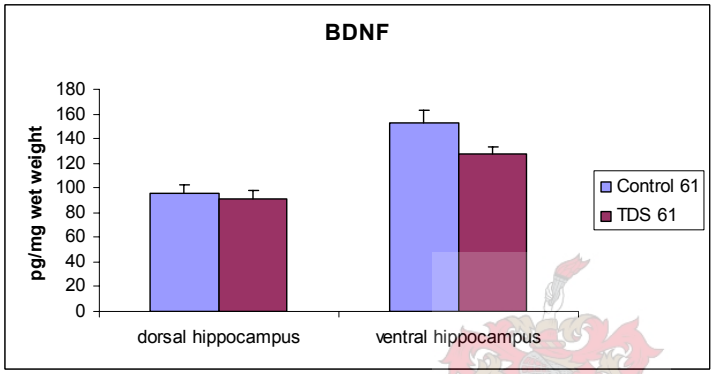

Figure 1c:

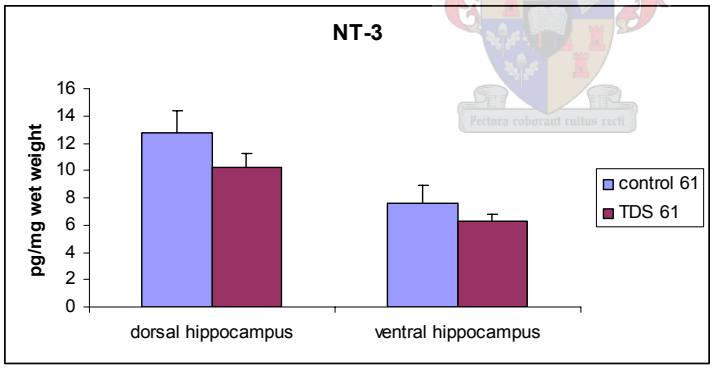

Figure 1: Histograms depicting (a) NGF-, (b) BDNF-, and (c) NT-3 protein concentrations in the dorsal and ventral hippocampus of control and traumatized animals. Values are expressed as mean ± SEM (n = 20) in pg/mg wet weight. * $p \leq 0.05$.

Chapter 5

Developmental trauma is associated with behavioral hyperarousal, altered HPA axis activity, and decreased hippocampal neurotrophin expression in the adult rat.

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Developmental trauma is associated with behavioral hyperarousal, altered HPA axis activity, and decreased hippocampal neurotrophin expression in the adult rat

Joachim D.K. Uys [1*], Lelanie Marais [1], Jacqueline Faure [2], Desiree Prevoo [3], Pieter Swart [3], Abdul H. Mohammed [4], Dan J. Stein [5], Willie M.U. Daniels [1]

1) MRC Unit for Anxiety and Stress Related Disorders, Department of Medical Physiology, University of Stellenbosch, Tygerberg, South Africa
2) Department of Physiological Sciences, University of Stellenbosch, Stellenbosch, South Africa
3) Department of Biochemistry, University of Stellenbosch, Stellenbosch, South Africa
4) Department of Neurotec, Karolinska Institutet, Stockholm, Sweden
5) Department of Psychiatry, University of Cape Town, Cape Town, South Africa

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* Corresponding author:
Joachim D.K. Uys
MRC Unit for Anxiety and Stress Related Disorders
Department of Medical Physiology
University of Stellenbosch
Tygerberg
7505
South Africa
Tel: +27 21 938 9531
Fax: +27 21 938 9476
Email: jduys@sun.ac.za

Keywords:
Dorsal hippocampus, ventral hippocampus, BDNF, NGF, NT-3, adrenal glands, time-dependent sensitization, early life stress.

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Abstract

Effects of early life trauma on adult behavioral responses, corticosterone concentration and levels of nerve growth factor, brain-derived neutrophic factor and neurotrophin-3 in hippocampus and frontal cortex were investigated. Traumatized animals showed an increase in rearing in both the elevated plus maze and open field after adult re-stress, higher basal levels of corticosterone, lower levels of BDNF in dorsal hippocampus, and lower levels of NT-3 in dorsal- and ventral hippocampus. Trauma related behavioral hyperarousal and altered HPA axis activity may be mediated by decreases in hippocampal neurotrophin expression.

Introduction

Early adverse experiences are known to be associated with later psychopathology, especially depression and anxiety disorders.[1] This association may be mediated by specific effects of early stressors on brain development. In particular, preclinical and clinical studies have suggested that trauma may be associated with loss of hippocampal neurons.[2] The hippocampus is the site of highest expression of the three members of the NGF family: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) [3], and there is data suggesting that they play a role in stress-mediated hippocampal atrophy.

Recently we showed that exposing young ("adolescent") animals (PND 28) to time-dependent sensitization (TDS), decreased glucocorticoid receptors in the dentate gyrus of the hippocampus and increased basal corticosterone levels in adulthood.[4] These changes were reversed by pretreatment with the serotonin selective re-uptake inhibitor (SSRI), escitalopram.[4] The present study focused on the effects of TDS during adolescence on behavior and neurotrophin levels in adulthood. We hypothesised that early trauma would lead to behavioral abnormalities in adulthood and these would be associated with reduced hippocampal neurotrophin levels

Materials and Methods

Trauma Procedure

All procedures were approved by the Committee for Ethical Animal Research. Male Sprague-Dawley rats (n=19) were housed in pairs under standard laboratory conditions. Based on the TDS model [5,6], the experimental group (n=10) was subjected to a triple stressor on PND 28 followed 7 days later (day 35) by the first re-stress session and 25 days later (day 60 = adulthood) by the second re-stress session.

Behavioral assessment

Behavioral assessment occurred 7 days after the last stress session when rats were 67 days old using the elevated plus maze (assessing time spent in closed and open arms, and rearing) and large open field (assessing crossing, and rearing) paradigms.

HPA Axis Assessment

Animals were decapitated and trunk blood collected 24 hours after behavioral assessment. Plasma adrenocorticotropin (ACTH) and corticosterone (CORT) concentrations were determined using RIA kits (IBL, Hamburg, Germany). Cytochrome P450 contents of microsomal preparations were determined following a slightly modified method of Omura and Sato.[7] The microsomes were placed in a 1 cm cuvette and reduced by sodium dithionate. Water saturated with carbon monoxide (CO) was added to the suspension to convert cytochrome P450 to carbon monoxide-binding pigment. This conversion was determined spectrophotometrically. The final concentration of P450 was calculated from CO difference spectra of the dithionite-reduced samples.

Neurotrophin levels

Dorsal and ventral hippocampi and frontal cortex were dissected on ice. Care was taken not to include any white matter in the dissections. After dissection, samples were snap frozen in liquid nitrogen until neurotrophin analysis. Dorsal and ventral hippocampi and frontal cortex samples were weighed and suspended in 400 µl lysis buffer. The samples were sonicated (45s), vortexed and centrifuged at 12000 rpm for 30 min at 4 ^{0}C and the supernatants were collected. Concentrations of NGF, BDNF and NT-3 were determined with E-Max ImmunoAssay system (Promega, Madison, Wisconsin, USA).

Results:

Behavior

There were no differences in time spent in the closed vs open arms in the elevated plus maze, or in crossing in the open field. However, TDS 67

animals showed a significant increase in rearing compared to controls in both paradigms ($p<.01$).

HPA Axis

There were no differences in ACTH between controls (20.24 ± 3.58 pg/ml) and TDS 67 (22.24 ±1.74 pg/ml) animals [$p = .63$]. There was, however, a significant effect in CORT (figure 1). TDS 67 animals had significantly higher levels of corticosterone compared to controls [$p < .05$]. Pooled concentrations of cytochrome P450 were 0.54nM/mg protein for controls and 0.11nM/mg protein for TDS 67 animals respectively.

Neurotrophin expression

There were no effects of TDS on NGF levels in the dorsal [$p = .21$], ventral hippocampus [$p = .07$] or frontal cortex [$p = .18$]. TDS 67 animals had significantly lower levels of BDNF in the dorsal hippocampus [$p < .05$]. There were no effects of TDS on BDNF levels in the ventral hippocampus [$p = .06$] or frontal cortex [$p = .98$]. TDS 67 animals had significantly lower levels of NT-3 in the dorsal hippocampus [$p < .05$] and ventral hippocampus [$p < .01$]. There were no differences in the frontal cortex between TDS 67 animals and controls [$p = .32$].

Discussion:

Animals subjected to TDS displayed some behavioral changes indicative of anxiety (increased rearing). Although no alterations were observed in plasma levels of ACTH, basal corticosterone levels in the TDS group were significantly higher than the control group of animals. This is consistent with animal models of depression, and clinical findings in human depression. A decrease in adrenal cytochrome P450 concentrations was found, and may reflect a dysfunction or a degree of adrenal insufficiency in these rats. Thus, the elevated basal corticosterone levels in TDS rats may result from chronic HPA-axis stimulation and/or ineffective negative feedback.

No changes were found in NGF protein expression between traumatized animals and controls. This is in contrast to previous studies where an increase in NGF protein expression and NGF mRNA was found in the hippocampus after early trauma, but these were studies of early maternal separation, rather than of later developmental trauma. Decrease in BDNF in the dorsal hippocampus, and a decrease in NT-3 in the dorsal and ventral hippocampus following trauma exposure during adolescence and subsequent adulthood re-stress, found here are consistent with basic and clinical studies [8] suggesting a role for neurotrophins in mediating response to maternal separation and depression. Ventral hippocampus had higher levels than dorsal hippocampus, consistent with functional differences along the dorsal-ventral axis.

To our knowledge, the current study is the first to show a decrease in NT-3 following adolescent trauma. Since NT-3 has been shown to contribute to neuron differentiation, synaptic plasticity and neuron survival,[9] the decrease observed in TDS rats may hinder neurogenesis and compromise hippocampal neurons. A chronic decrease in hippocampal neurotrophic (NT-3) support, coupled with elevated levels of corticosterone, could lead to hippocampal neuronal loss, and increase hippocampal vulnerability to later stressors leading to development of behavioral abnormalities.[2,10]

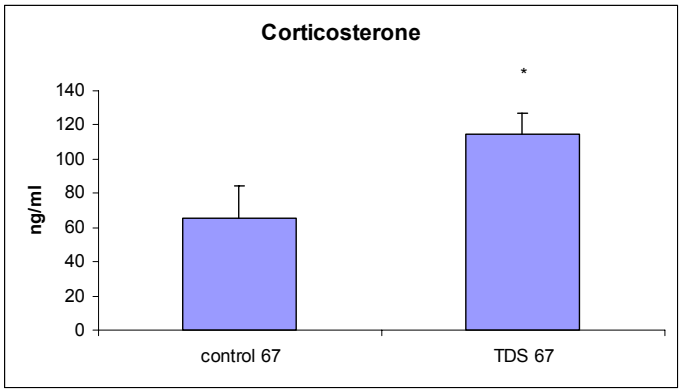

Figure 1: Plasma corticosterone levels of control and traumatized animals.Values are expressed as mean ± SEM in ng/ml. Traumatized animals were subjected to a triple stressor on PND 28 (restraint stress, swim stress and ether inhalation), and to swim stress alone on PND 35 and PND 60. Trunk blood was collected PND 68. * $p \leq 0.05$.

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Chapter 6

Effects of early trauma and adult re-stress on subsequent hippocampal and plasma neurotrophin expression and phosphoinositide-3 kinase signaling proteins

Submitted *BMC Neuroscience*

Effects of early trauma and adult re-stress on subsequent hippocampal and plasma neurotrophin expression and phosphoinositide-3 kinase signaling proteins

Joachim D.K. Uys [1*], Lorren Fairbairn [1], Dan J. Stein [2], Willie M.U. Daniels [1]

1) MRC Unit for Anxiety and Stress Related Disorders, Department of Biomedical Sciences, Division of Medical Physiology, University of Stellenbosch, Tygerberg, South Africa
2) Department of Psychiatry, University of Cape Town, Cape Town, South Africa

Email addresses:

JDKU: jduys@sun.ac.za
LF: lf@sun.ac.za
DJS: dan.stein@curie.uct.ac.za
WMUD: wd@sun.ac.za

* Corresponding author:
Joachim D.K. Uys
MRC Unit for Anxiety and Stress Related Disorders
Department of Biomedical Sciences
Division of Medical Physiology
University of Stellenbosch
Tygerberg
7505
South Africa
Tel: +27 21 938 9531
Fax: +27 21 938 9476
Email: jduys@sun.ac.za

Abstract

Background

Early adverse experience is associated with later psychopathology, especially depression and anxiety disorders. This association may be due in part to the effects of stressors on hippocampal development, mediated by abnormalities in neurotrophin levels. The purpose of the study was to investigate the effects of early life trauma and adult re-stress on the levels of nerve growth factor, brain-derived neurotrophic factor and neurotrophin-3 in the dorsal, ventral hippocampus and plasma. In addition, signaling proteins of the phosphoinositide-3 kinase pathway were also measured.

Young rats (n=31) were subjected to a triple stressor on post-natal day 28 followed 7 days later by the first re-stress session and 25 days later (post-natal day 60 = adulthood) by the second re-stress session. Brain tissue (dorsal and ventral hippocampi) and plasma were collected on post-natal day 68 for neurotrophin and protein determinations.

Results

Compared to controls, traumatized animals had significantly lower levels of brain-derived neurotrophic factor in the dorsal hippocampus, and lower levels of neurotrophin-3 in the dorsal- and ventral hippocampus compared to controls. The expression levels of phosphoinositide-3 kinase (PI-3 kinase), protein kinase B (PKB), phosphatase and tensin homologue (PTEN), phospho-forkhead and phospho-AFX did not differ significantly between traumatized and control groups. There were also no significant differences in neurotrophin levels when measured in the plasma.

Conclusions

Our data suggest that adolescent trauma with re-stress may lead to decreases in neurotrophin expression in the hippocampus. Alterations in neurotrophin concentrations may therefore be responsible for abnormal brain development and function. The changes in neurotrophin levels were not reflected in the phosphoinositide-3 kinase pathway, suggesting a role for other transduction proteins to participate in its downstream effects. The dissociation between plasma and hippocampal neurotrophin levels cautioned against using parameters in the blood to indicate abnormalities in the brain.

Background

The hippocampus is one of the few areas where production of neurons occurs throughout the life of animals, including humans [1]. Hippocampal neurogenesis can be influenced by several different environmental factors [2; 3]. Environmental stressors, including prenatal, physical and psychosocial stressors [3; 4], may decrease neurogenesis and so contribute to structural changes in the hippocampus. Preclinical and clinical studies have suggested that severe stress or trauma may be associated with loss of hippocampal neurons [5; 6; 7]. In particular, early life trauma has been shown to be associated with the development of psychopathology, especially depression and anxiety disorders [8; 9]. Some of these disorders may be characterized by reduced hippocampal volume [10; 11; 12].

The hippocampus is the site of highest expression of the three members of the neurotrophin family: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) [13]. Neurotrophins are essential for the optimal maintenance of neurons, utilizing a range of mechanisms for example, upregulation of the BDNF pathway enhances the survival and function of neurons [14], the cAMP-CREB-BDNF pathway induces expression of the anti-apoptotic protein bcl-2 [15], NGF promotes growth, plasticity and survival of neurons [16; 17; 18].
Consistent with this important role is evidence that neurotrophins such as BDNF play a role in mediating antidepressant effects [19; 20; 21]. Preclinically, electroconvulsive seizures increased BDNF in the hippocampus of rats [22], and bilateral infusion of BDNF in the hippocampus of rats exposed to the learned helplessness or forced swim tests paradigms had antidepressant effects [19]. In humans diagnosed with remitted major depressive disorder, there was a decreased response of serum BDNF levels after tryptophan depletion when compared to healthy controls [23].

There are a number of inconsistencies in the literature regarding the role of neurotrophins in stress-related abnormalities. NGF levels were decreased in

animal models of depression [24], but increased in models of neonatal stimulation [25] and environmental enrichment [26]. Furthermore, significant increases in NGF levels have been shown after cold water swim stress [27] and NGF is suggested to activate the hypothalamic-pituitary-adrenal (HPA) axis. Some controversy remains regarding the role of NT-3 in stress-induced psychopathology [28]. It has been reported that 8 hours of immobilisation stress reduces NT-3 mRNA levels in the hippocampus [29], while others have reported an increase in NT-3 mRNA levels in the hippocampus and dentate gyrus following chronic but not acute immobilisation stress [30]. It is possible that one explanation lies in the difference between NT-3 expression after single vs repeated stress.

NGF, BDNF and NT-3 bind to tropomyosin related kinase (Trk) receptors [31; 32]. NGF binds to TrkA, BDNF to TrkB and NT-3 to TrkC and to a lesser extent also TrkB [32]. Trk receptors are phosphorylated at tyrosine residues and activate three major pathways, the Ras/MAPK cascade, inositol triphosphate (IP_3) dependent Ca^{2+} release and the phosphoinositide-3 kinase (PI-3 kinase) [32; 33].
In order to understand the underlying mechanism, a focus on PI-3 kinase would be useful. The PI-3 kinase pathway is crucial for cell survival [33; 34] where it links proteins such as poly(adenosine diphosphate-ribose) polymerase (PARP) and Forkhead proteins [35; 36]. PARP is activated during apoptosis when it is cleaved by apoptotic proteases such as caspase 3 and 7. This pathway is therefore associated with cell death [37]. On the other hand, phosphorylation of forkhead proteins can be mediated via neurotrophin signaling and thereby play a role in neurotrophin mediated cell survival [35]. Phosphatase and tensin homologue deleted on chromosome 10 (PTEN), is a phosphatase that has been shown to be a negative regulator of the PI-3 kinase/Akt pathway and to inhibit cell survival [38]. It has the ability to remove phosphates from lipids and helps regulate PI-3 kinase action [39].

There is a growing literature documenting the effects of early life adverse events. The majority of this work has focused on stressful situations around the immediate postnatal period [40; 41; 42], and our group has recently

focused on the effects of stress in adolescent animals (PND 28) plus adult re-stress. This work demonstrated structural and functional changes in the hippocampus as evidenced by a decrease in glucocorticoid receptors in the dentate gyrus and an increase in basal corticosterone levels in adulthood [43]. These changes were reversed by pre-treatment with the serotonin selective re-uptake inhibitor (SSRI), escitalopram [43]. Nevertheless, hippocampal neurotrophin levels were unchanged 24 hours after the adulthood re-stress [44]. Whilst decreases in BDNF mRNA only 4 hours after a corticosterone injection has been reported [45], it seems that a longer time period is required for BDNF protein levels to be affected. The aim of the present study was therefore to investigate whether changes in hippocampal neurotrophin levels and PI-3 kinase signaling proteins would occur 8 days after the last re-stress.

Results

Neurotrophin expression in hippocampal tissue

NGF protein levels

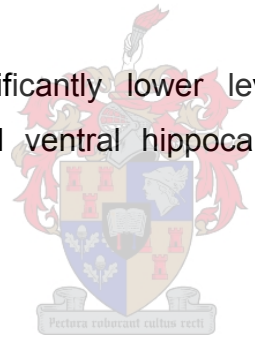
On PND 68 there were no effects of TDS on NGF levels in the dorsal ($p = .21$) or ventral hippocampus ($p = .07$) (figure 1).

BDNF protein levels

TDS 68 animals had significantly lower levels of BDNF in the dorsal hippocampus compared to controls ($p \leq .05$). A numerical difference in ventral hippocampal BDNF levels between controls and TDS 68 animals did not reach statistical significance ($p = .06$) (figure 2).

NT-3 protein levels

TDS 68 animals had significantly lower levels of NT-3 in the dorsal hippocampus ($p \leq .05$) and ventral hippocampus ($p \leq .01$) compared to controls (figure 3) on PND 68.



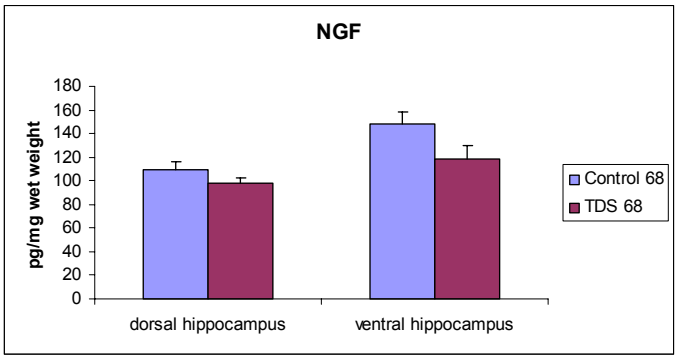


Figure 1: Histograms depicting NGF protein concentrations in the dorsal and ventral hippocampus of control and traumatized animals. Values are expressed as mean ± SEM (n = 19) in pg/mg wet weight. * $p \leq 0.05$.

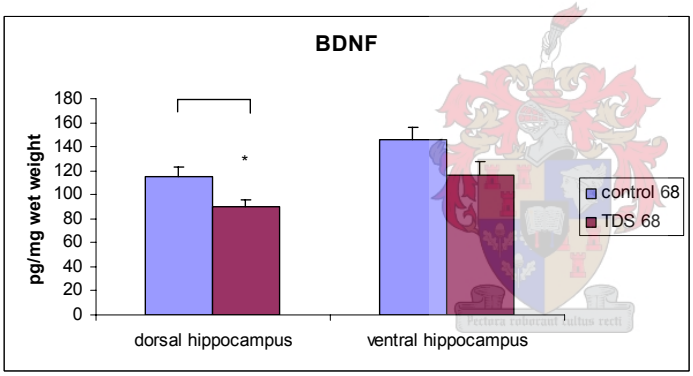


Figure 2: Histograms depicting BDNF protein concentrations in the dorsal and ventral hippocampus of control and traumatized animals. Values are expressed as mean ± SEM (n = 19) in pg/mg wet weight. * $p \leq 0.05$.

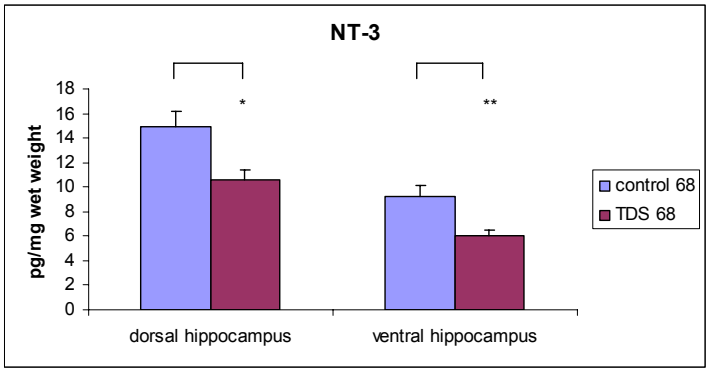


Figure 3: Histograms depicting NT-3 protein concentrations in the dorsal and ventral hippocampus of control and traumatized animals. Values are expressed as mean ± SEM (n = 19) in pg/mg wet weight. * $p \leq 0.05$, ** $p \leq 0.01$.

Stressor control experiment

There were no effects of either the “triple stressor day + re-stress day 35” or “stress day 60 only” on BDNF levels in the dorsal [$F(2,34) = 0.287$, $p = .75$], ventral hippocampus [$F(2,34) = 0.99$, $p = .38$] or on NT-3 levels in the dorsal [$F(2,34) = 2.022$, $p = .15$], ventral hippocampus [$F(2,34) = 0.356$, $p = .70$] or on NGF levels in the dorsal [$F(2,34) = 0.186$, $p = .83$] or ventral hippocampus [$F(2,34) = 1.371$, $p = .27$].

Neurotrophin expression in plasma

BDNF and NT-3 plasma protein levels

There were no effects of TDS on BDNF ($p = .40$) or NT-3 ($p = .23$) protein levels in the plasma.

Western Blots

There were no differences between control and TDS 68 animals in protein levels (figure 4) of PI-3 kinase in the dorsal ($p = .91$) or ventral hippocampus ($p = .96$), PKB/Akt in the dorsal ($p = .53$) or ventral hippocampus ($p = .31$), PTEN in the dorsal ($p = .89$) or ventral hippocampus ($p = .81$), or phospho-forkhead ($p = .08$) and phospho-AFX ($p = .32$) in the ventral hippocampus. In addition, there were no significant differences in β-tubulin in any of the blots, indicating equal protein loading.

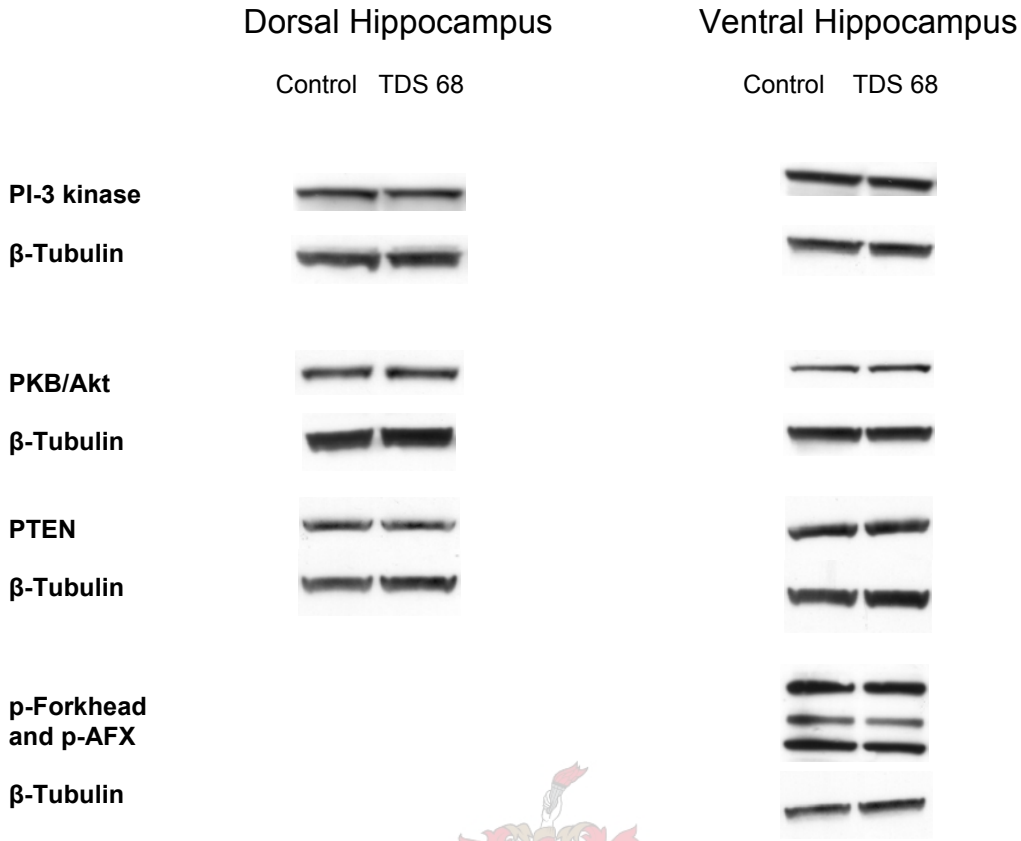


Figure 4: Representative Western Blot images of PI-3 kinase, PKB/Akt, PTEN, phospho-Forkhead and phospho-AFX in the hippocampus. In addition, β-tubulin expression did not differ between control and traumatized animals, which indicates equal protein loading (n = 12).

Discussion

Early adverse events have been suggested to contribute to psychopathology later in life [8; 9]. A number of studies have focused on identifying the molecular mediators of this process [46; 47]. Here we were able to show that early trauma plus later re-stress, but not early or late stress alone, were associated with a decrease in BDNF levels in the dorsal hippocampus, and a decrease in NT-3 in the dorsal and ventral hippocampus following trauma exposure during adolescence and subsequent adulthood re-stress. This is consistent with basic studies and indirectly with clinical studies, which provide evidence for the role of neurotrophins in mediating depression [18; 19; 21] and response to stress [29; 48]. Other animal models of early life stress, where rats and mice were exposed to maternal separation also show decreased levels in BDNF in the hippocampus [49; 50].

Furthermore, we demonstrate a differential regulation of dorsal and ventral hippocampal BDNF levels, with the ventral having higher levels than the dorsal hippocampus. Available evidence indicates that the hippocampus is functionally heterogeneous along the dorsal-ventral axis. The hippocampus is known to have an important role in memory, as well as in emotional processing. The dorsal hippocampus appears to be preferentially involved in spatial learning, while the ventral hippocampus is crucial for anxiety-like behaviour [51]. For example, electrolytic lesioning of the ventral but not the dorsal hippocampus results in reduced freezing behavior to context, tone conditioned stimuli as well as decreased escape latency from enclosed arms of the elevated T-maze, indicating less anxious behaviour [52].

In the current study we have shown a decrease in NT-3 following adolescent trauma. Since NT-3 has been shown to contribute to neuron differentiation [53], synaptic plasticity [54] and neuron survival [18], the decrease observed in TDS rats may hinder neurogenesis and compromise hippocampal neurons. Indeed, NT-3 is known to prevent neurotoxic-induced degeneration of adult brain noradrenergic neurons [55]. Therefore a chronic decrease in hippocampal neurotrophic (NT-3) support, coupled with elevated levels of

corticosterone, could lead to hippocampal neuronal loss [56], and increase the vulnerability of the organism to later stressors [7; 57].

No changes were found in NGF brain protein expression between traumatized animals and controls. This is in contrast to previous studies where an increase in NGF protein expression [58] and NGF mRNA [59] was found in the hippocampus after early trauma. However, these were studies of early maternal separation (PND 3 or PND 9 and PND 16), whereas the current study focused on adolescent trauma.

We also measured plasma BDNF and NT-3 levels to see whether the changes in hippocampal neurotrophin levels were reflected in the blood. No changes were evident in the plasma BDNF or NT-3 levels of traumatized animals when compared to controls. This is in contrast to a recent clinical study that showed a decrease in plasma BDNF levels in patients diagnosed with depression [60]. It is therefore possible that a dissociation between brain and plasma neurotrophin levels exists in our animals, unlike human subjects.

No changes were found in PI-3 kinase, PKB/Akt, PTEN, phospho-Forkhead and phospho-AFX in the areas examined in the hippocampus. There is the possibility that adolescent trauma would alter the expression of other proteins in the Ras/MAPK cascade or IP_3 dependent Ca^{2+} release pathways, since these are also activated by Trk receptors [32] and susceptible to both psychosocial [61] and physical stressors [62].

Conclusion

In summary, adolescent trauma causes a decrease in hippocampal BDNF and NT-3 but not in total levels of certain PI-3K pathway constituents. In addition, the experiments done here demonstrate the value of focusing on multiple stressors over time. In this study, controls that were subjected to trauma but not to re-stress as adults did not show changes in neurotrophin levels. Further studies will focus on repetitive stressors and other intracellular signaling proteins to advance our understanding about the interaction of these parameters and the associated molecular changes.

Methods

Animals

Male Sprague-Dawley rats (n = 68) were studied. Animals were housed in pairs under standard laboratory conditions (12/12 hours light/dark cycle, food and water ad libitum) in the central animal research facility of the University of Stellenbosch. All procedures were approved by the Committee for Ethical Animal Research.

Trauma procedure

The trauma procedure is based on the TDS model where a single exposure of sequential stressors of escalating severity is followed by a subsequent exposure to one of the stressors 7 days later [63; 64; 65]. The rationale is that the frequency of exposure to situational reminders contributes to the maintenance over time of fear-related behavioral disturbances. Young rats were subjected to a triple stressor on PND 28 followed 7 days later (day 35) by the first re-stress session and 25 days later (day 60 = adulthood) by the second re-stress session. The triple stressor consisted of being placed in a plexiglass restrainer for 2hrs with the tail-gate adjusted to keep the rat well contained without impairing circulation to the limbs. Immediately thereafter, the rats were placed individually in 18 cm of ambient water (25°C) in a perspex swim tank and forced to swim for 20 minutes. After gently drying the rat with a soft towel and 30 minutes recovery, each rat was then immediately exposed to ether vapors until loss of consciousness. The animals were then placed in their home cages for further experimentation.

Experimental design

For the both the neurotrophin and western blot experiments, animals were divided into two experimental groups (brain and plasma neurotrophin experiment total n = 19; western blot experiment total n = 12): control 68 and TDS 68 (8 days post second re-stress).

In addition to the above mentioned groups, a stressor control experiment was done (n = 37) where animals were divided into 3 groups (group 1: control, group 2: triple stressor day 28 + re-stress day 35, group 3: stress day 60 only)

to measure neurotrophin levels. This control experiment was intended to show that the complete combination of stressors (ie triple stressor day 28 + re-stress day 35 + second re-stress day 60) were needed to affect the neurotrophin levels.

Tissue dissection and blood collection

The dorsal and ventral hippocampi were dissected on ice. Care was taken not to include any white matter in the dissections. After dissection, samples were snap frozen in liquid nitrogen until neurotrophin analysis. For plasma determinations, trunk blood was collected in chilled EDTA tubes after decapitation. Blood was centrifuged at 4 degrees for 5min at 3000rpm and plasma stored in liquid nitrogen until analysis.

Measurement of neurotrophin levels

The dorsal and ventral hippocampi samples were weighed and suspended in 400 µl lysis buffer [137mM NaCl, 20mM Tris-HCl (pH 8.0), 1% NP40, 10% glycerol, 1mM PMSF, 10 µg/ml aprotinin, 1 µg/ml leupeptin and 0.5mM sodium vanadate]. The samples were sonicated (45s), vortexed and centrifuged at 12000 rpm for 30 min at 4 ^{0}C and the supernatants were collected.

The concentrations of NGF (tissue only), BDNF and NT-3 were determined using the E-Max ImmunoAssay system (Promega, Madison). Standard 96-well flat-bottom Greiner ELISA plates were incubated with carbonate coating buffer containing either polyclonal anti-NGF, monoclonal anti-BDNF or polyclonal anti-Human NT-3 overnight at 4 ^{0}C. The next day the plates were blocked with 1 x block and sample buffer for 1 h at room temperature. Serial dilutions of known amounts of NGF, BDNF and NT-3 ranging from 500 to 0 pg/ml (NGF and BDNF) and 300 to 0 pg/ml (for NT-3) were performed in duplicate for the standard curve for each set of rat tissue. For both the standards and samples, 100 µl was added to each well in duplicate and incubated for 6 h (NGF and NT-3) or 2 h (BDNF) at room temperature. Plasma samples (BDNF and NT-3) were assayed in triplicate. The wells were then incubated with a secondary monoclonal anti-NGF, secondary monoclonal anti-NT-3 (overnight at 4 ^{0}C) or anti-human BDNF polyclonal antibody (2 h

room temperature). Then the wells were incubated with anti-rat IgG (NGF), anti-IgY (BDNF) or anti-mouse IgG (NT-3) conjugated to HRP for 2.5 h (NGF and NT-3) or 1 h (BDNF) at room temperature. A TMB solution was used to develop color in the wells for 10 min (NGF and BDNF) or 15 min (NT-3) at room temperature. The reaction was stopped with the addition of 1 N HCl to the wells. The absorbance was read at A_{450} within 30 min on a Bio-Rad benchmark microplate reader with Microplate Manager version 5.2 software. Data are expressed as pg/mg wet weight for the tissue samples and pg/ml for the plasma samples.

Western Blots

Dorsal and ventral hippocampi of control and traumatized rats were lysed in buffer containing 50mM Tris-HCl (pH 7.4), 1% sodium deoxycholate, 50mM sodium fluoride, 20um zinc chloride, 1mM sodium vanadate, 0.5 mM PMSF, 2ug/ml aprotinin and 2ug/ml leupeptin. After homogenizing, samples were centrifuged at 14000 rpm for 30 minutes at 4 deg. Protein concentration was determined from the cleared supernatant using a Bradford reagent kit (Bio-Rad). Samples were aliquotted, boiled in MOPS XT sample buffer (Bio-Rad) at 95 deg for 5 min with XT reducing agent (Bio-Rad) and frozen in at -80 until analysis.

Pectora roborant cultus recti

Equal concentrations of protein (30-50ug) were loaded in each well and proteins were separated by SDS-PAGE on a Criterion XT system using pre-cast 4-12% Bis-Tris gels (Bio-Rad). The proteins were transferred to a nitrocellulose membrane and blocked overnight in Detector Block solution (KPL) at 4°C. The following day the membranes, blotted for PI-3 kinase, were incubated with anti-PI-3 kinase (1:2000, Upstate) for 1 h at room temperature. In addition, other membranes were blocked for 1 h at room temperature in Detector Block solution (KPL) and incubated overnight at 4°C with anti-Akt/PKB, anti-PTEN or anti-phospho-Forkhead (all 1:1000, Cell Signaling). Anti-phospho-Forkhead also recognizes anti-phospho-AFX. After incubation the membranes were washed in PBS-Tween 20 (0.1%) and incubated with either an anti-mouse or anti-Rabbit HRP linked antibody (Amersham) for 1 h at room temperature. After extensive washing the protein bands were

visualized by chemiluminesence using LumiGlo Reserve (KPL) and put on ECL Hyperfilm (Amersham). After visualization the membranes were stripped and incubated overnight with anti-β-Tubulin (1:1000, Cell Signaling) to determine equal protein loading.

Statistical analysis

Data were analyzed with either an independent Students t-test or ANOVA (stressor control experiment) with SPSS version 11.0 software. Protein bands were imaged using a densitometer and total number of pixels counted using UN-SCAN-IT gel version 5.1 (Silk Scientific, USA). Differences were considered significant if $p \leq .05$.

Authors' contributions

JDKU conceived the study, participated in the design, carried out the Western Blot experiments and part of the ELISA experiments, did the data analysis and contributed to interpretation of the data.
LF carried out part of the ELISA experiments, helped with acquisition of data and to draft the manuscript.
DJS participated in the design, made substantial intellectual contribution to the interpretation of the data and helped to draft the manuscript.
WMUD participated in the design, made substantial intellectual contribution to the interpretation of the data and helped to draft the manuscript.
All authors read and approved the final manuscript.

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Chapter 7

Neuroproteomics: Relevance to Anxiety Disorders

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Neuroproteomics: Relevance to Anxiety Disorders

Joachim D.K. Uys [1], M.Sc., Dan J. Stein [2], M.D., Ph.D, Willie M.U. Daniels [1], Ph.D

[1] MRC Unit for Stress and Anxiety Disorders, Laboratory for Neuroproteomics, Department of Biomedical Sciences, Division of Medical Physiology, University of Stellenbosch, Tygerberg, South Africa
[2] Department of Psychiatry, University of Cape Town, Cape Town, South Africa

* Corresponding author:
E-mail: jduys@sun.ac.za
Tel: +27 21 938 9531
Fax: +27 21 938 9072

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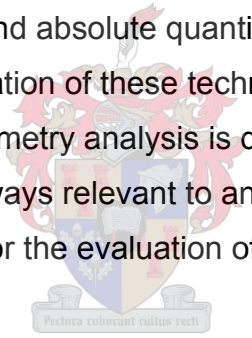
Abstract

Despite advances in the treatment of anxiety disorders, there is a need for medications with greater efficacy and fewer side-effects. Advances in techniques to facilitate high throughput, mass analysis of proteins potentially allows for new drug targets, with a shift in focus from membrane receptor proteins and enzymes of neurotransmitter metabolism to molecules in intracellular signal transduction and other pathways. A computerized literature search was done to collect studies on recently developed proteomic techniques and their application in psychiatric research. Particular techniques such as 2-dimensional electrophoresis (2D-SDS PAGE), 2-dimensional differential gel electrophoresis (2D-DiGE), isotope-coded affinity tags (ICAT) and isotope tags for relative and absolute quantification (iTRAQ) are reviewed. In addition, combination of these techniques with MALDI-TOF/TOF and ESI-Q-TOF mass spectrometry analysis is discussed in relation to possible novel signaling pathways relevant to anxiety disorders, and to the development of bio-markers for the evaluation of these conditions.



Introduction

Anxiety disorders are the most prevalent of the psychiatric disorders, and are associated with significant disability and social costs [1]. Fortunately there have been significant advances in the treatment of many of these conditions, including generalized anxiety disorder, panic disorder, posttraumatic stress disorder, and social anxiety disorder [2]. Nevertheless, many patients either do not respond to pharmacotherapy, or suffer significant adverse events, and there is therefore a need for new medications.

The development of medications for anxiety disorders has been a serendipitous process. Early anxiolytic agents were subsequently found to act on a limited number of amino acid and monoamine receptors. More recently, advances in cellular biology have led to a shift in focus from such receptors to intracellular signaling pathways [3; 4; 5]. There is growing data demonstrating that agents commonly used in the treatment of anxiety disorders alter mRNA expression of a range of signaling pathways [6].

With advances in our knowledge of the human genome, there are a range of potential targets for the development of new agents for the treatment of anxiety disorders. Nevertheless, each gene may lead to multiple protein targets. Furthermore genomics has been accompanied by advances in proteomics. In the current review we focus on this field, reviewing recent methodologies, and considering their application to the field of anxiety disorders.

What is proteomics?

One of the surprising findings of the human genome project was the relatively low number of human genes [7]. An explanation for the complexity of human biology lies in exploring the multiple products that emerge from the genome; the so-called transcriptome, or the entire complement of mRNA transcripts transcribed. Furthermore, there is subsequent modification of proteins; with post-translational phosphorylation, glycosylation, acetylation, nitrosation, poly-

ADP- ribosylation, ubiquination, farnesylation, sulfation, linkage to glycophosphatidylinositol anchors, and SUMOylation. The 300 or more such modifications reported to date affect protein stability, localization, binding interactions and function [8].

Proteomics encompasses the study of the entire protein population on a large scale to obtain a global view protein networks and their function in cellular processes and pathology [9; 10; 11]. The power of this approach has been illustrated in the identification of members of signal transduction pathways, of their post-translational modifications [12], and of their interactions in complex and network formation [13] over time [14].

Central to the methodology of obtaining proteomic data is the use of mass spectrometry [15]. Isolated proteins are digested enzymatically to yield a variety of peptide fragments. These are then further separated and subjected to high-sensitivity mass spectrometry (MS). This is followed by the identification of the proteins through matching the MS spectra to a large-scale peptide/protein database, such as Swiss-Prot or from the National Center for Biotechnology Information (NCBI). Advances in MS technology have made it possible to identify proteins in complex mixtures accurately and rapidly [16].

Current proteomic strategies

2D Gel Electrophoresis and 2D-DiGE

Two-dimensional gel electrophoresis (2D-SDS PAGE) coupled to mass spectrometry has been widely employed in the study of proteins in biological samples [17]. In this method, proteins are separated in the first dimension on pH followed by separation on molecular weight in the second dimension (see figure 1). Subsequently the spots that are differentially expressed (for example between control and patient) are excised from the gel and trypsinated (in-gel digestion), yielding peptides. These peptides are subjected to MS and followed by matching the MS spectra to a peptide/protein database.

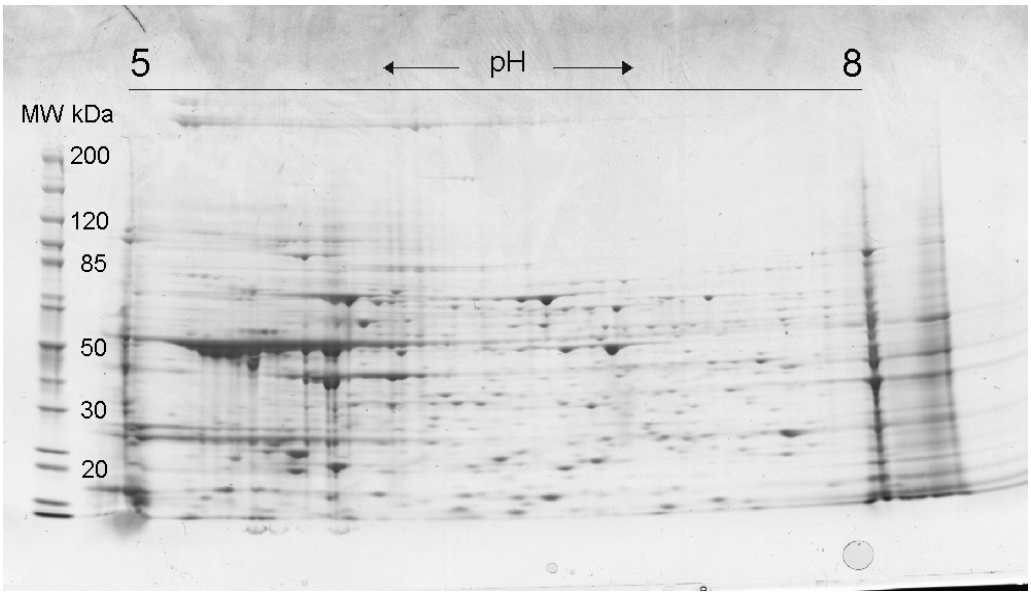
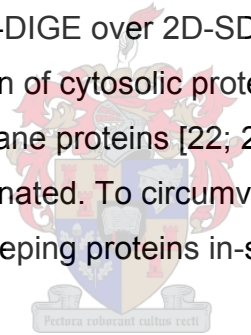


Figure 1: In two-dimensional gel electrophoresis, proteins are separated in the first dimension on pH, followed by separation on molecular weight (MW) in the second dimension. Here, proteins are separated on pH 5 to 8 in the first dimension. The marker on the left shows a molecular weight range from 200 kDa to 20 kDa for the second dimension separation.

A variant of 2D-SDS PAGE is 2-Dimensional Differential Gel Electrophoresis (2D-DiGE). In this method samples from two different conditions are each labeled with a fluorophore, Cy3 and Cy5. The samples are then combined and run on one gel instead of two gels, which minimizes gel-to-gel variation [18; 19]. Scanning of the gels at different wavelengths then allows for the images to be overlaid. In addition, the use of fluorescent dyes to label proteins, increases sensitivity and thereby enhancing identification of proteins which are expressed at very low levels in the cell. A third dye (Cy2) can be used as an internal standard by pooling an equal amount of all the samples. Every spot would therefore be represented in the standard, increasing quantification and the power of statistical analysis between gels.

It has been shown that up to 1000 protein spots can be adequately resolved [20] and post-translational modifications can be detected on 2D gels [21]. Despite the advantages of 2D-DIGE over 2D-SDS PAGE, there are still some disadvantages. The expression of cytosolic proteins is favoured by 2D electrophoresis above membrane proteins [22; 23] and the problem of gel-to-gel variation is not totally eliminated. To circumvent some of these problems, attention has been given to keeping proteins in-solution.



Isotope-coded affinity tags (ICAT)

One such relative quantification method where proteins are kept in-solution is by tagging the proteins with stable isotopes, also known as isotope-coded affinity tags (ICAT). In short, samples from a control and experimental condition are each tagged with stable isotope, combined, digested and subjected to MS analysis. The newest ICAT reagent has a thiol-specific reactive group adjacent to an alkyl linker, which contains either nine [12C] or nine [13C] atoms, resulting in a mass difference of 9 daltons between the control and experimental/patient sample [24]. By labeling each sample with its own isotope, the relative ratio of the peptides of both conditions can be compared in the same MS run. An added advantage of ICAT labeling is the fact that the reagent labels only cysteine containing peptides, thereby decreasing sample complexity. The ratios between control and experimental/patient peptides are calculated by commercially available

software such as ProICAT (Applied Biosystems).

Isotope tags for relative and absolute quantification (iTRAQ)
Recently another relative and absolute (when using internal standards) quantification method for labeling proteins in solution has been developed, called iTRAQ [25] (Applied Biosystems Inc, CA). Instead of labeling cysteine containing peptides, labeling is done on primary amines, which allows tagging of most tryptic peptides. In addition, whereas ICAT labels only two different conditions, iTRAQ allows up to four different conditions, making it ideal for pharmacology experiments (control + no treatment, control + treatment, experimental + no treatment, experimental + treatment). The quantification of the tags is performed in MS/MS (see next section), where the relative abundance of the four product ions (114, 115, 116, 117) is measured after cleavage from the tags. Here too the ratios are calculated by commercially available software such as ProQuant (Applied Biosystems Inc, CA) or freeware such as i-Tracker [26].

Mass Spectrometry and databases in proteomic analysis

Advances in mass spectrometry have made it possible to not only determine peptide masses but also peptide sequences – allowing greater accuracy in identification of proteins. After digesting proteins with an enzyme, such as trypsin, the resulting peptides can either be spotted on a plate for matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) analysis or be injected into the mass spectrometer via high-performance liquid chromatography (HPLC) when using electrospray ionization quadrupole time-of-flight (ESI-Q-TOF) mass spectrometry. The peptides are ionized by either laser or applying an electrical charge when emerging from the HPLC column. In the TOF module, the time is measured that it takes for ions to travel through the flight tube. Where MALDI-TOF mass spectrometry uses the weight of peptides [27; 28], also termed a peptide mass fingerprint (PMF), ESI-Q-TOF mass spectrometry uses peptide sequences to determine identification [29; 30]. Recently, development of MALDI-TOF-TOF mass spectrometry, allows peptides [31; 32] as well as intact proteins [33], to be sequenced using MALDI

technology. As noted earlier, the data (PMF or peptide sequences) generated from the mass spectrometer are then matched against a protein database to identify possible peptide-protein matches through the use of different algorithms such as Mascot [30] or Sequest [34].

Application of proteomic strategies in animal models of psychiatric disorders

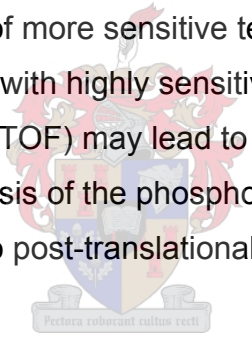
A number of these proteomic techniques have been applied to study the nervous system in general (neuroproteomics) and to animal models of stress in particular [35]. Combining 2D-SDS PAGE and MALDI-TOF mass spectrometry, the researchers identified 21 hippocampal proteins, from different subcellular locations in an animal model of repeated psychosocial stress. These included proteins involved in intracellular signaling, synaptic plasticity, cytoskeleton regulation and energy metabolism. In addition, the protein changes observed were different from a single exposure as opposed to multiple exposures [35]. Some of these data are consistent with those found in previous studies, for example the increase in HSC71 is also evident in restraint-water immersion stress [36]. Proteomic analysis of hippocampal proteins in this model of repeated psychosocial stress using more sensitive 2D-DiGE or iTRAQ tagging may provide additional data on differentially expressed proteins.

The hyperanxious (HAB-M) and hypoanxious (LAB-M) CD1 mouse lines have been proposed as a model of anxiety. Proteomic analysis of selected brain areas in these animals (hypothalamus, amygdala, and motor cortex), using 2D SDS PAGE, found that the enzyme glyoxalase-I (Glx1) was 5 times higher in LAB-M animals compared with HAB-M animals [37]. This is reminiscent of data from an animal model for Alzheimer disease, where the Glx1 gene was upregulated [38]. In addition, a link has been found between the Glx1 gene and depression [39]. Future directions could include the validation of this protein in other animal models of anxiety disorders [40].

In another animal experiment using 2D-SDS PAGE, chronic administration of either venlafaxine or fluoxetine to adult rats was shown to differentially

regulate at least 33 cytosolic proteins in the hippocampus [41]. Mass spectrometry analysis identified proteins that included, but were not limited to, those associated with neurogenesis (eg. insulin-like growth factor and glia maturation factor) and synaptic plasticity (eg. Ras-related protein 4a, Ras-related protein 1b, heat shock protein 10). This is consistent with previous studies where fluoxetine prevented the increase in heat shock protein after phencyclidine (PCP) and dizocilpine (MK801) induced neurotoxicity [42]. It would be interesting to see if similar results would be obtained using the 4 isobaric tags of iTRAQ in a stress experiment with and without pharmacological treatment.

These initial studies demonstrate the promise of applying proteomic techniques to validated animal models of anxiety. Whether the differentially expressed proteins are also applicable as a biomarker in a clinical setting remains to be seen. The use of more sensitive techniques, such as 2D-DiGE and iTRAQ tagging combined with highly sensitive tandem mass spectrometry (MALDI-TOF-TOF and ESI-Q-TOF) may lead to the discovery of additional proteins. In addition, the analysis of the phosphoproteome of this animal model may provide insight into post-translational modifications of proteins.



Application of proteomic strategies in the clinical setting

The use of neuroproteomics (or neuromics) to investigate protein expression and changes related to psychiatric disorders is at an early stage [43]. Initial work has begun in schizophrenia, Alzheimer's disease, and Parkinson's disease [reviewed in 43]. In postmortem frontal cortical tissue, patients with either schizophrenia, bipolar disorder or major depressive disorder, showed changes in proteins associated with synaptic plasticity (glial fibrillary acidic protein) and enzymatic proteins (fructose biphosphate aldolase) seen with 2D SDS PAGE and mass spectrometry [44].

Cerebral spinal fluid (CSF) proteomic analysis of patients diagnosed with either chronic fatigue syndrome (CFS), Persian Gulf War Illness (PGI), or fibromyalgia showed that CFS and PGI shared 20 proteins not detectable in

the control sample [45]. These included alpha-1-macroglobulin, and amyloid precursor-like protein 1. In addition, 62 of 115 proteins were newly described in terms of a possible role in psychiatric disorder. Future steps would be investigating and validating these proteins in animal models. In addition, using techniques like prefractionation of the cerobral spinal fluid may increase the identification of additional proteins. Also, using an in-solution tagging approach such as ICAT or iTRAQ may aid in identifying proteins which are differenitally expressed between patients.

In a genetic microarray study, differential gene expression signatures were identified in leucocytes of traumatized patients who were subsequently diagnosed with posttraumatic stress disorder (PTSD) compared to those who remained well [46]. This approach shows the potential of mass analysis techniques in the context of anxiety disorders. A similar protein approach, using 2D-DiGE or iTRAQ, could potentially be used to study the differential CSF proteome expression in patients diagnosed with an anxiety disorder vs healthy controls.

Conclusion

There have been significant developments in proteomics in general, and there is potential to extend this work to the study of basic models of anxiety, as well as to clinical populations. A detailed understanding of the psychobiology of psychiatric disorders will ultimately require a comprehensive understanding of the relevant proteins that underpin symptoms. Current methods are available for exploring the neuroproteome. For instance, analysis of the mouse hippocampal membrane proteome has revealed more than 1,685 proteins using a highly sensitive Fourier transform mass spectrometer [47]. In addition, the initial pilot studies of the Human Proteome Organisation (HUPO) Brain Proteome Project (BPP) of human and mouse brain samples, is in an advanced stage [48]. This promises further elucidation of neural proteins and their possible functions.

Further development of advanced protein technologies is still needed. Comparison between a Q-TOF and a highly sensitive Fourier transform mass spectrometer in the analysis of the mouse hippocampal membrane proteome revealed 862 proteins (Q-TOF) vs 1685 (Fourier transform) [47]. This illustrates how more sensitive technologies could aid in the identification of a greater number of proteins. Currently the databases used for peptide/protein searches do not contain all species. In addition, very large data files are generated by high-throughput proteomic experiments, causing a “bottle-neck” during analysis, since often peptide sequences have to be manually inspected to avoid false positives.

There is a growing evidence-base, however, which suggests that differential analysis of expressed proteins can aid in the discovery of biomarkers and of novel therapeutic targets. Ultimately this may lead to the development of more specific pharmacological agents for the treatment of psychiatric disorders, including the anxiety disorders.

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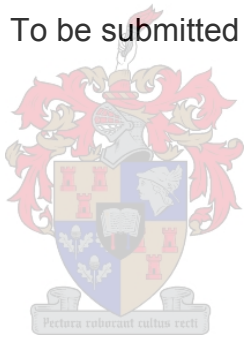
Chapter 8

Early life trauma leads to large scale hippocampal cellular distress in adulthood – a proteomic approach.

To be submitted



Early life trauma leads to large scale hippocampal cellular distress in adulthood – a proteomic approach.

Joachim D.K. Uys [1] , Suzél M Hattingh [1] , Dan J. Stein [2] , Willie M.U. Daniels [1*]

[1] Department of Biomedical Sciences, Division of Medical Physiology, University of Stellenbosch, Cape Town, South Africa

[2] Department of Psychiatry, University of Cape Town, Cape Town, South Africa

* Corresponding author:

E-mail: wd@sun.ac.za

Tel: +27 21 938 9385

Fax: +27 21 938 9476

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Abstract

Early life trauma is associated with psychopathology in adulthood. These pathologies include depression and anxiety disorders. This association may be due in part to the effects of trauma on hippocampal development and protein expression. The purpose of the study was to investigate the effects of early life trauma and adult re-stress on ventral hippocampal protein expression using commercially available protein arrays and 2D-SDS PAGE combined with liquid chromatography tandem mass spectrometry (LC-MS/MS).

Adolescent rats (n=19) were subjected to a triple stressor on post-natal day 28 followed 7 days later by the first re-stress session and 25 days later (post-natal day 60 = adulthood) by the second re-stress session. Ventral hippocampi were collected on post-natal day 68 for protein expression determinations.

Compared to controls, traumatized animals showed an increase in Ca^{2+} homeostatic proteins, dysregulated signaling pathways and energy metabolism enzymes, cytoskeletal protein changes, a decrease in neuroplasticity regulators, energy metabolism enzymes and an increase in apoptotic initiator proteins. These include, but were not limited to, S-100B, phospho-JNK, ARNO, Caspase 9, 10 and 12, MAP-1b, syntaxin and DRP-2. A total number of 40 proteins were differentially regulated by adolescent trauma.

These results indicate the extensive impact of adolescent trauma on adult brain development and may point to novel targets for pharmacological treatment.

Introduction

Early life trauma is known to cause behavioural abnormalities later in life (Heim and Nemeroff, 2001; Kendler et al., 1992). These include mood and anxiety disorders such as depression and posttraumtic stress disorder (PTSD). An important brain area implicated in the pathology of these disorders is the hippocampus (Duman et al., 1997; Duman et al., 1999; McEwen, 1999). For example, a reduced hippocampal volume has been documented in patients diagnosed with depression (Neumeister et al., 2005) and PTSD (Kitayama et al., 2005). Support for the involvement of an abnormal hippocampus in these disorders comes from basic studies where animals, subjected to different stressors (physical, psychosocial, and prenatal stressors) show decreased hippocampal neurogenesis, which may contribute to the observed structural changes in the hippocampus (Czeh et al., 2002; Lemaire et al., 2000). However the underlying molecular mechanisms responsible for mediating the effects of early life trauma, remains unclear.

We have recently shown that animals which were subjected to adolescent trauma and adulthood re-stress have higher basal corticosterone levels and decreased glucocorticoid receptors in the dentate gyrus of the hippocampus (Uys et al., 2006b). In addition, traumatized animals also showed a decrease in BDNF in the dorsal hippocampus and a decrease in NT-3 in the dorsal and ventral hippocampus. This trauma-induced decrease in neurotrophins was accompanied by behavioural hyperarousal (Uys et al., 2006a) but not by changes in the proteins, phosphoinositide-3 kinase (PI-3 kinase), protein kinase B (PKB), phosphatase and tensin homologue (PTEN), phospho-forkhead and phospho-AFX (Uys et al., submitted). However, numerous other cell signaling and cell proliferation pathways may participate in regulating trauma-induced changes. For instance, the Ras/MAPK cascade or inositol triphosphate (IP_3) dependent Ca^{2+} release pathways are activated by physical stressors (Herbert et al., 2005) or psychosocial stress (Pardon et al., 2005).

In order to investigate changes in protein expression after early life trauma on a global scale we employed a systems biology approach by using proteomic methods – protein arrays and 2D-SDS PAGE combined with liquid chromatography tandem mass spectrometry (LC-MS/MS). Here we were able to show that animals subjected to early life trauma show signs of cellular stress, cell cycle arrest, initiation of apoptotic mechanisms and cell signaling dysregulation.

Methods

Animals

Male Sprague-Dawley rats (N = 19) were studied. Animals were housed in pairs under standard laboratory conditions (12/12 hours light/dark cycle, food and water *ad libitum*) in the AALAC acredited central animal research facility of the University of Stellenbosch. All procedures were approved by the Committee for Ethical Animal Research.

Trauma procedure

The trauma procedure is based on the TDS model where a single exposure of sequential stressors of escalating severity is followed by a subsequent exposure to one of the stressors 7 days later (Liberzon et al., 1997; Yehuda and Antelman, 1993; Harvey et al., 2003). The rationale is that the frequency of exposure to situational reminders contributes to the maintenance over time of fear-related behavioral disturbances. Young rats were subjected to a triple stressor on post-natal day (PND) 28 followed 7 days later (PND day 35) by the first re-stress session and 25 days later (PND day 60 = adulthood) by the second re-stress session. The triple stressor consisted of being placed in a plexiglass restrainer for 2hrs with the tail-gate adjusted to keep the rat well contained without impairing circulation to the limbs. Immediately thereafter, the rats were placed individually in 18 cm of ambient water (25°C) in a perspex swim tank and forced to swim for 20 minutes. After gently drying the rat with a soft towel and 30 minutes recovery, each rat was exposed to ether vapors until loss of consciousness. The animals were then placed in their home cages for further experimentation. Re-stress sessions consisted of 20 minutes forced swim.

Experimental design

Animals were divided into two experimental groups: control 68 and TDS 68 (8 days post second re-stress). On PND 68 animals were decapitated and the ventral hippocampi were dissected on ice. Care was taken not to include any

white matter in the dissections. After dissection, samples were snap frozen in liquid nitrogen until analysis.

Protein array

A commercially available antibody-based protein array (Panorama Cell Signaling Array, Sigma, USA) was used to determine relative differential expression of proteins between control 68 and TDS 68 animals. The array contained 224 different antibodies spotted in duplicate on nitrocellulose glass slides. The antibodies occured in 32 sub-arrays each containing duplicate spots of 7 antibodies, as well as a single positive (BSA labeled with Cy3 and Cy5) and negative control spot. Each sample is labeled with a different Cy dye (Cy3 or Cy5), combined and applied simultaneously on the array.

Sample preparation for protein array

Samples were prepared as recommended by the array manufacturer. In short, ventral hippocampi were homogenized on ice in extraction buffer with protease inhibitors, phosphatase inhibitors and benzonase. Protein extracts were clarified by centrifugation (10 000 rpm for 20 seconds) and the protein concentration of the supernatant was measured by the Bradford method. The samples were diluted to 1mg/ml and 1ml of the supernatant was used for labeling with Cy3 or Cy5 dyes (Amersham Biosciences). Excess dye was removed using Sigma Spin columns and the protein concentration of the eluate was measured by the Bradford method. The Cy3 and Cy5 concentration were measured by reading the absorbance of Cy3 at 552nM, dividing the value by 0.15 and Cy5 at 650nM and dividing the vaule by 0.25. The dye to protein molar ratio was calculated to ensure a ratio > 2. Control 68 and TDS 68 samples were mixed in equal protein concentrations (4ug protein each) and incubated on the array for 45 minutes at room temperature. In addition, each control 68 and TDS 68 sample were labeled with both Cy3 and Cy5 and mixed with the counterpart labeled with the other dye. This ensured the experiment is fully controlled and doubly tested. After extensive washing in phosphate buffered saline (PBS)-Tween 20 (0.1%), slides were air dried and prepared for scanning.

Protein array data analysis and normalization

Protein array slides were scanned with a GenePix 4000A scanner (Molecular Devices, USA) and images were captured with GenePix Pro version 6 with scan resolution at 10µm. After background subtraction, values were normalized to a log base 2 value, where a log value (M-value) of 1 equaled a 2-fold increase and log value of -1 a 2-fold decrease, relative to control. A global LOESS (also known as LOWESS) normalization (Smyth and Speed, 2003) was used to perform a location and scale normalization of the arrays (R, Bioconductor software). This was necessary, since the two Cy dyes differ in fluorescence intensity and labeling efficiency, thereby correcting for intensity dependent dye-bias. A small number of housekeeping proteins were included on the array. It was decided not to fit the normalization curve to the housekeeping proteins as there are very few of them and they displayed considerable variation. Furthermore, they also do not cover the entire intensity range of the arrays. The underlying assumption we made by using the global LOESS approach is that the majority of proteins will not show differential expression with respect to the control (see figure 1). We feel that this is a reasonable assumption for our arrays. An exploratory analysis of the data confirmed this. S100 beta, which was upregulated on the protein array, was selected for further confirmation by Western Blot. In addition, Bcl-2 expression was also determined by Western Blot.

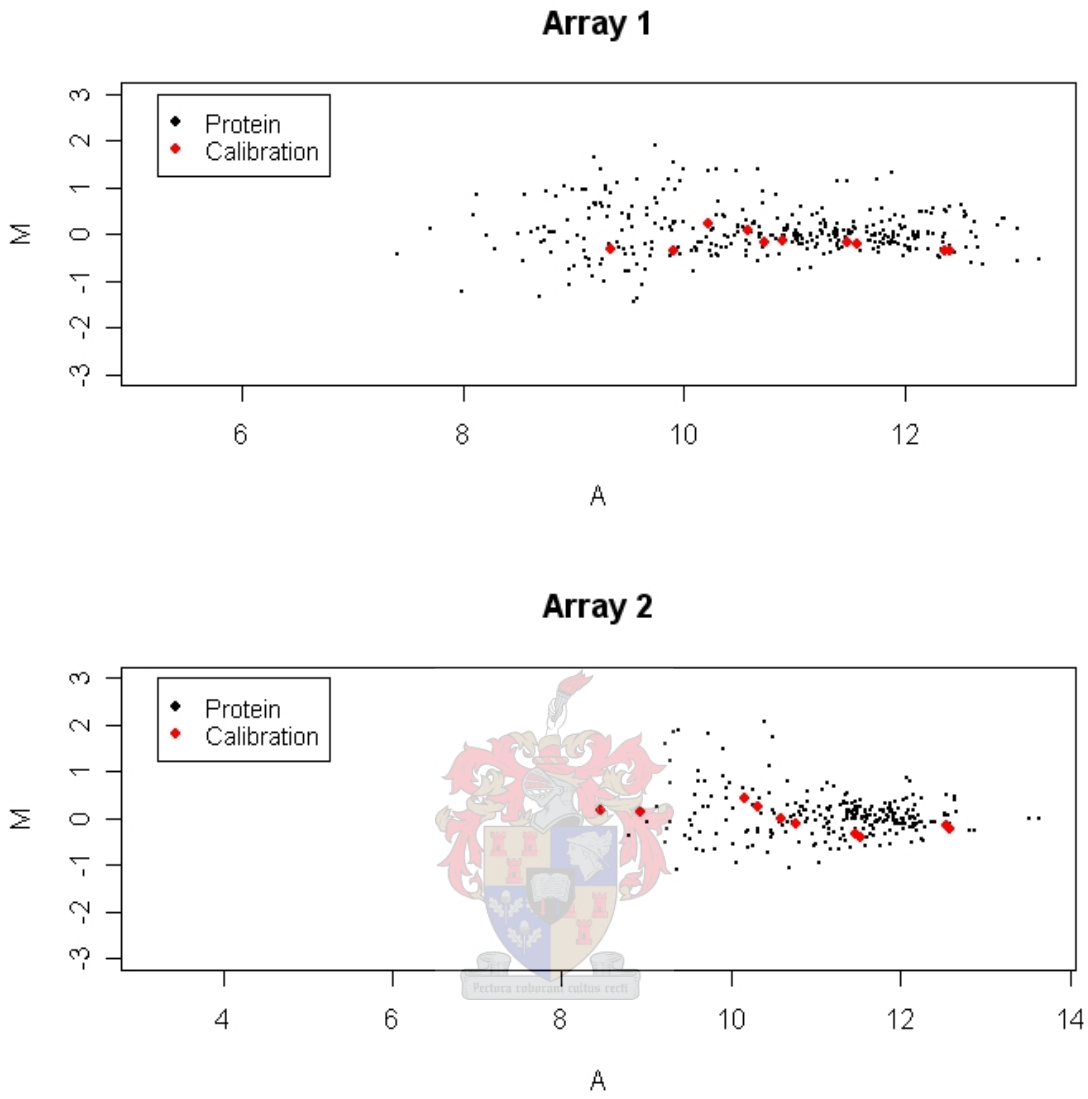


Figure 1: A global LOESS normalization was used to perform a location and scale normalization of the arrays. The majority of proteins did not show differential expression with respect to the control. Array 1 and Array 2 represents the Cy3 and Cy5 dye-swap between control 68 and TDS 68.

Western Blot

Ventral hippocampi of control 68 and TDS 68 rats were lysed in buffer containing 50mM Tris-HCl (pH 7.4), 1% sodium deoxycholate, 0.5 % NP-40, 50mM sodium fluoride, 20μM zinc chloride, 1mM sodium vanadate, 0.5 mM PMSF, 2μg/ml aprotinin and 2μg/ml leupeptin. After homogenizing, samples were centrifuged at 14000 rpm for 30 min at 4°C. Protein concentration was determined from the cleared supernatant using a Bradford reagent kit (Bio-Rad). Samples were aliquotted, boiled in MOPS XT sample buffer (Bio-Rad) at 95 °C for 5 min with XT reducing agent (Bio-Rad) and frozen at -80 °C until analysis.

Equal concentrations of protein (20μg) were loaded in each well and proteins were separated by SDS-PAGE on a Criterion XT system using pre-cast 4-12% Bis-Tris gels (Bio-Rad). The proteins were transferred to a PVDF membrane and blocked overnight in 5% Milk/PBS-Tween 20 (0.1%) at 4°C. The following day the membranes were incubated with either anti-S100 beta (1:1000, Abcam, United Kingdom) or anti-Bcl-2 (1:1000, Stressgen, USA) for 1 h at room temperature. After incubation the membranes were washed in PBS-Tween 20 (0.1%) and incubated with either an anti-mouse or anti-Rabbit HRP linked antibody (Amersham) for 1 h at room temperature. After extensive washing the protein bands were visualized by chemiluminesence using LumiGlo Reserve (KPL) and put on ECL Hyperfilm (Amersham). After visualization the membranes were stripped and incubated overnight with anti-β-Tubulin (1:1000, Cell Signaling) to determine equal protein loading.

Western Blot data analysis

Protein bands were imaged using a GS-800 calibrated densitometer (Bio-Rad, Hercules, CA, USA) and total optical density of pixels counted using Quantity 1 software, version 4.5.2 (Bio-Rad, Hercules, CA, USA). Data were normalized for β-Tubulin levels, expressed relative to controls and analyzed with an independent Students t-test with SPSS version 11.0 software. Differences were considered significant if $p \leq .05$.

Sample preparation for 2D electrophoresis

Ventral hippocampi were fractionated into cytosolic fractions using a commercially avaliable kit (ProteoExtract Subcellular Proteome Extraction kit, Calbiochem). Lipids, salts and detergents were removed from the cytosolic proteins by precipitation using the ReadyPrep 2D clean-up kit (Bio-Rad, Hercules, CA, USA). Precipitated samples were re-suspended in 2D sample/Rehydration buffer (Bio-Rad, Hercules, CA, USA) and protein concentrations were measured using the RC/DC protein assay (Bio-Rad, Hercules, CA, USA).

2D electrophoresis

Isoelectric focusing was carried out on a 11cm immobilised pH strip (5-8, Bio-Rad, Hercules, CA, USA). Strips were rehydrated for 12 hours with 200ug protein in 200ul of 2D sample/Rehydration buffer (Bio-Rad, Hercules, CA, USA). Focusing was carried out at 20 °C for 40 000 Vh with a maximum of 8000 V in a Protean IEF Cell (Bio-Rad, Hercules, CA, USA). Strips, from the same batch, were then incubated for 15 min with gentle shaking in equilibration buffer I (6 M Urea, 2% w/v SDS, 2% DTT, 20% glycerol, 375 mM Tris pH 8.8, Bio-Rad, Hercules, CA, USA) followed by 15 min incubation in equilibration buffer II (6 M Urea, 2% w/v SDS, 2.5% w/v iodoacetamide, 20% glycerol, 375 mM Tris pH 8.8, Bio-Rad, Hercules, CA, USA). Control 68 and TDS 68 strips were run in triplicate on pre-cast 4-12% Criterion XT Bis-Tris gels, from the same batch, in Criterion XT electrophoresis cells (Bio-Rad, Hercules, CA, USA) for 42 minutes. Gels were fixed in 7% v/v glacial acetic acid and 40% v/v methanol for 1 hour followed by 2 hour staining in Coomassie Colloidal Blue (Sigma, USA). Gels were destained overnight in 25 % v/v methanol.

Data Analysis for 2D electrophoresis

Images were captured on a GS-800 calibrated densitometer (Bio-Rad, Hercules, CA, USA) and were analyzed with PDQuest 8 Advanced software (Bio-Rad, Hercules, CA, USA). A number of spots that were significantly ($p \leq .05$) differentially expressed between control 68 and TDS 68 as assesed by

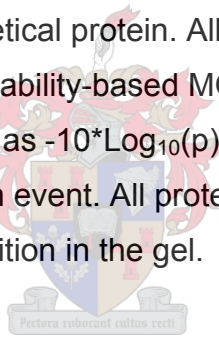
PDQuest with a T-test, were selected for spot excision and protein identification by LC-MS/MS analysis.

Protein Identification using LC-MS/MS

Excised gel spots were destained, reduced, alkylated, digested with trypsin and the resulting peptides extracted using the Micromass MassPrep Station (Waters Technologies, Manchester, U.K.) running the standard digestion protocol supplied by the manufacturer. The tryptic peptides were transferred to a cooled second 96-well microtitre plate and if necessary, stored at –20^{o}C. The 96-well microtitre plate containing the extracted tryptic peptides was transferred to a Micromass modular CapLC and autosampler system (Waters Technologies, Manchester, U.K.). A 6.4 μl aliquot of extract was mixed with 13.6 μl of 0.1% formic acid and loaded onto a 0.5 cm LC Packings C18 5 μm 100Å 300 μm i.d μ-precolumn cartridge (Dionex Corporation, Sunnyvale, CA, U.S.A). Flushing the column with solution A (95% water, 4.9% acetonitrile, 0.1% formic acid) desalted the bound peptides before a linear gradient of solution B (4.9% water, 95% acetonitrile,0.1% formic acid) at a flow rate of 200 nlmin^{-1} eluted the peptides for further resolution on a 15 cm LC Packings C18 5 μm 5Å 75 μm i.d. PepMap analytical column (Dionex Corporation, Sunnyvale, CA, U.S.A). Gradient separations were 0.10 min (95%A:5%B), 3 min (95%A:5%B), 31 min (55%A:45%B), 35min (20%A:80%B), 37min (20%A: 80%B), 38 min (95%A:5%B), 47 min (95%A:5%B).

The eluted peptides were analysed on a Micromass QTof Global Ultima mass spectrometer (Waters Technologies, Manchester, U.K.) fitted with a nano-LC sprayer with an applied capillary voltage of 3.5 kV. The instrument was calibrated against a collisionally induced decomposition (CID) spectrum of the doubly charged precursor ion of GFP. A calibration was accepted when the error obtained on all subsequent acquisitions was <50 ppm. Sensitivity is assessed by the detection of a 500 fmol injection of GFP, with a base peak signal:noise ratio of >20:1 on the doubly charged ion. Both the sensitivity and calibration were checked at regular intervals during the analysis.

The instrument was operated in data dependent acquisition (DDA) mode over the mass/charge (*m/z*) range of 50-2000. During the DDA analysis, both MS and tandem mass spectrometry (CID) was performed on the most intense peptides as they eluted from the column. The uninterpreted MS/MS data was processed using the Micromass ProteinLynx software package, which converted the raw data into .pkl files for use with the Mascot search engine (www.matrixscience.com). The data files were submitted online and the MS/MS files were searched against the Swiss-Prot database with Mammalia as subspecies. Search parameters specified were: carbamidomethylated at cysteine residues, oxidized at methionine residues, one missed trypsin cleavage allowed, a 0.2 Da tolerance against the database-generated theoretical peptide and product ion masses and a minimum of 1 matched peptide. If there was only 1 matched peptide, the sequence was manually inspected to determine a minimum of 4 consecutive Y-ions matched and a unique match against the theoretical protein. All protein identifications were significant according to the probability-based MOWSE scores (Mascot scores in table 2), which were reported as $-10*\text{Log}_{10}(p)$, where p is the probability that the observed match is a random event. All proteins identified were in the expected size based on the position in the gel.



Results

Protein array:

A cut-off limit of 50% up- or down-regulated was applied to the array proteins and 25 were found to be differentially expressed between control 68 and TDS 68 animals using this criterium. The proteins were grouped into functional groups (see Table 1).

MAP-1b, Cytokeratin, S-100b, Histone 3 (acetylated on lysine 9), phospho-JNK, phospho-serine-FAK, syntaxin, p34 (cdc2), chk1, calcineurin, desmin, caspase 9, caspase 10, caspase 12, c-Myc and $p57^{Kip2}$ were increased in TDS 68 animals.

In contrast, cdc25C, cdk7, cdk6, ARNO, $p16^{INK4a}$, c-Abl, β-tubulin polyglutamylated, neurofilament (200 kDa heavy chain) and phospho-Pyk2, showed decreases compared to controls.

Western Blot analysis:

In order to verify the protein array data, western blot analysis was performed on two randomly selected proteins. They were S-100B that showed increased expression in traumatized animals and Bcl-2 whose concentration was not affected by the trauma. These protein array observations were therefore confirmed (see figure 2).

Table 1: Up- or down regulated proteins on the array. A cut-off limit of 50% increase or decrease compared to controls was applied. Results are expressed relative to control.

Protein	M-value	Fold increase or decrease	Functional Group
S-100B	1.37	2.58	Ca^{2+} homeostasis/ signaling
Pyk2-pTyr881	-0.64	- 1.56	Ca^{2+} homeostasis/ signaling
JNK activated di-phosphopeptide	1.12	2.17	Ca^{2+} homeostasis/ signaling
Calcineurin	0.88	1.84	Ca^{2+} homeostasis/ signaling
ARNO	-1.08	- 2.11	neuroplasticity
c-Abl	-1.02	- 1.95	neuroplasticity
FAK-pSer910	1.11	2.16	apoptosis
Caspase 10	0.83	1.78	apoptosis
Caspase 9	0.82	1.77	apoptosis
Caspase 12	0.65	1.57	apoptosis
β-Tubulin polyglutamylated	-0.77	- 1.71	cytoskeletal
Desmin	0.84	1.79	cytoskeleton
Cytokeratin	1.64	3.12	cytoskeleton
Neurofilament 200	-0.67	- 1.59	cytoskeleton
Syntaxin	0.96	1.93	vesicular docking and fusion
MAP-1b	1.90	3.73	Structural membrane
Cdc25C	- 1.44	- 2.71	cell cycle
chk1	0.94	1.91	cell cycle
cdk7	-1.34	- 2.53	cell cycle
cdk6	-1.22	- 2.33	cell cycle
$p16^{INK4a}$	-1.07	- 2.03	cell cycle
$p57^{(Kip2)}$	0.59	1.51	cell cycle
p34 (cdc2)	0.95	1.92	cell cycle
c-Myc (mono)	0.67	1.59	nuclear
Acetyl Histone 3-Ac-Lys 9	1.37	2.58	nuclear

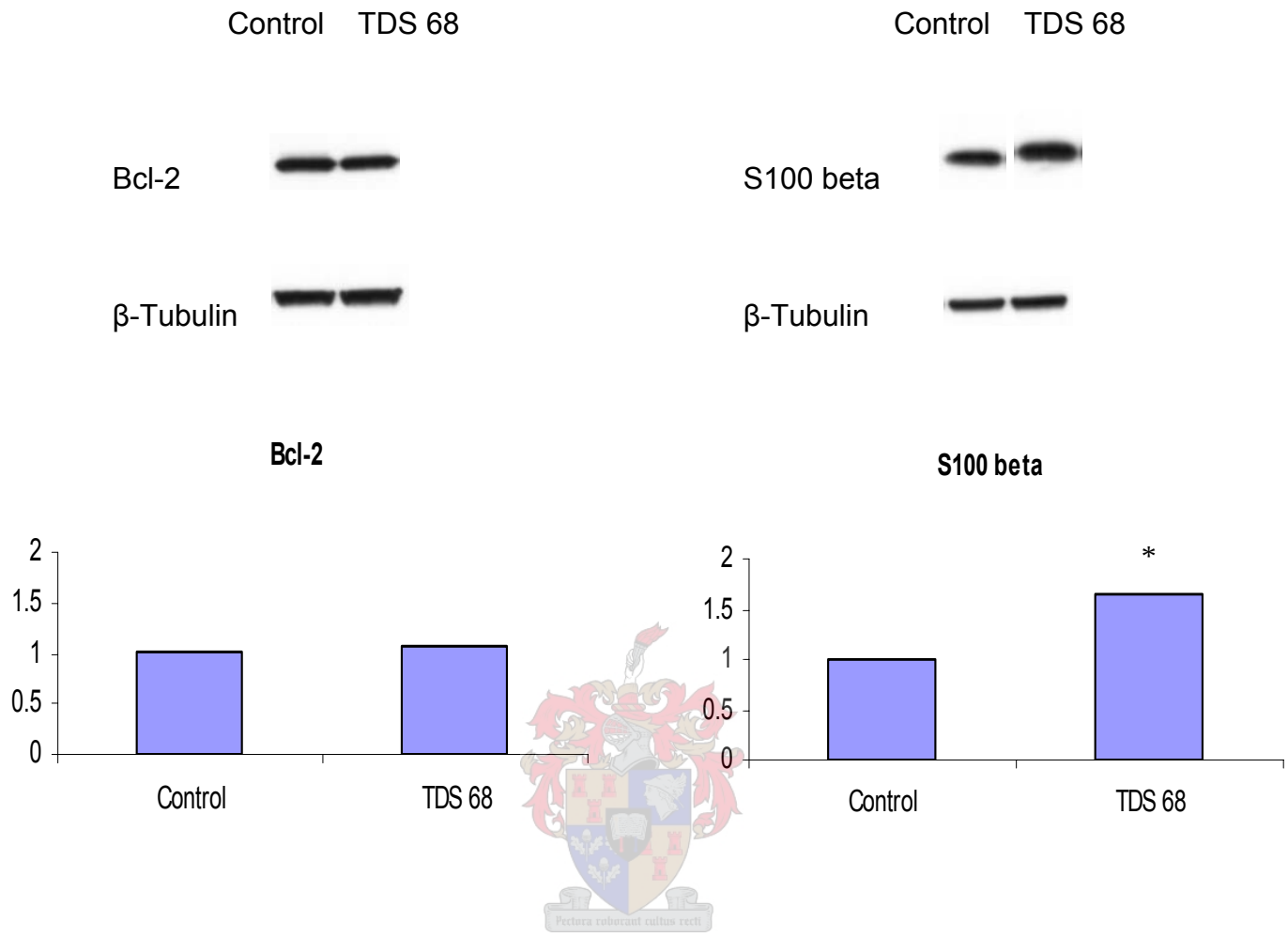


Figure 2: Histograms depicting Bcl-2 and S100 beta protein expression in the ventral hippocampus of animals subjected to early life trauma. Densitometric data were normalized for β-Tubulin levels and expressed relative to controls (n = 11, * p ≤ .05). Representative images of the respective blots are shown above.

2D-SDS PAGE with LC-MS/MS:

Numerous proteins were found to be significantly differentially expressed between control 68 and TDS 68 animals. Proteins that were upregulated in traumatized animals were synaphin-1, ubiquitin carboxyl-terminal hydrolase 1, pyruvate dehydrogenase and cofilin-1. Downregulated proteins were MAP kinase kinase 1 (MEK 1), neuron-specific calcium-binding protein hippocalcin, dihydropyrimidinase-related protein 2 (DRP-2), guanine deaminase, neuronal protein NP25 (Transgelin-3), ATPase, ADP-ribosylarginine hydrolase, vacuolar ATP synthase, transferrin, creatine kinase and eukaryotic translation initiation factor 5A-1. The proteins were also grouped into functional groups (see Table 2, figure 3a and 3b).

Table 2: Data represents the differentially expressed proteins between control 68 and TDS 68 animals. Only those proteins whose concentrations changes significantly are reported ($p \leq .05$, T-test). The SSP number is assigned by PDQuest software.

	Mr (Da)	pI	MASCOT score	Protein name	Swiss Prot Accession Number	% Sequence Coverage	No of peptides	Fold increase or decrease	Functional group
5403	43648	6.20	82	MAP kinase kinase 1 (MEK 1)	Q01986	13%	4	-3.35	Ca^{2+} homeostasis / signaling
1002	22396	4.87	52	Neuron-specific calcium-binding protein hippocalcin	P84076	4%	1	-1.81	Ca^{2+} homeostasis / signaling
5702	62638	5.95	243	Dihydropyrimidinase-related protein 2 (DRP-2)	P47942	18	8	-2.01	neuroplasticity
4502	51554	5.56	547	Guanine deaminase	Q9WTT6	41%	17	-1.29	neuroplasticity
8004	22627	6.84	265	Transgelin-3 (Neuronal protein NP25)	Q9R1Q8	41%	10	-1.51	cytoskeleton
0002	15499	5.06	68	Complexin -2 (Synaphin-1)	P84087	19%	4	2.00	vesicular docking and fusion
6002	18618	8.26	153	Cofilin-1	P45592	46%	5	1.26	cell cycle
4803	68700	5.41	239	similar to ATPase (predicted from NCBI Blast sequence search)	P31404 (Bovine)	22%	9	-2.23	energy metabolism

4303	40220	5.53	49	ADP-ribosylarginine hydrolase	Q02589	9%	3	-1.50	energy metabolism
3803	68567	5.62	169	Vacuolar ATP synthase	P50516	10%	5	-4.74	energy metabolism
3305	39336	5.94	344	Pyruvate dehydrogenase	P49432	31%	13	1.57	energy metabolism
4401	42970	5.33	583	Creatine kinase	P07335	55%	22	-1.56	energy metabolism
1003	16918	5.08	135	Eukaryotic translation initiation factor 5A-1	Q3T1J1	33%	7	-1.19	cell proliferation
2102	25165	5.14	228	Ubiquitin carboxyl-terminal hydrolase 1 (Ubiquitin thioesterase)	Q00981	55%	8	1.47	protein degradation
8801	78538	6.94	119	Transferrin	P12346-00-00-00	6%	4	-1.67	iron transport

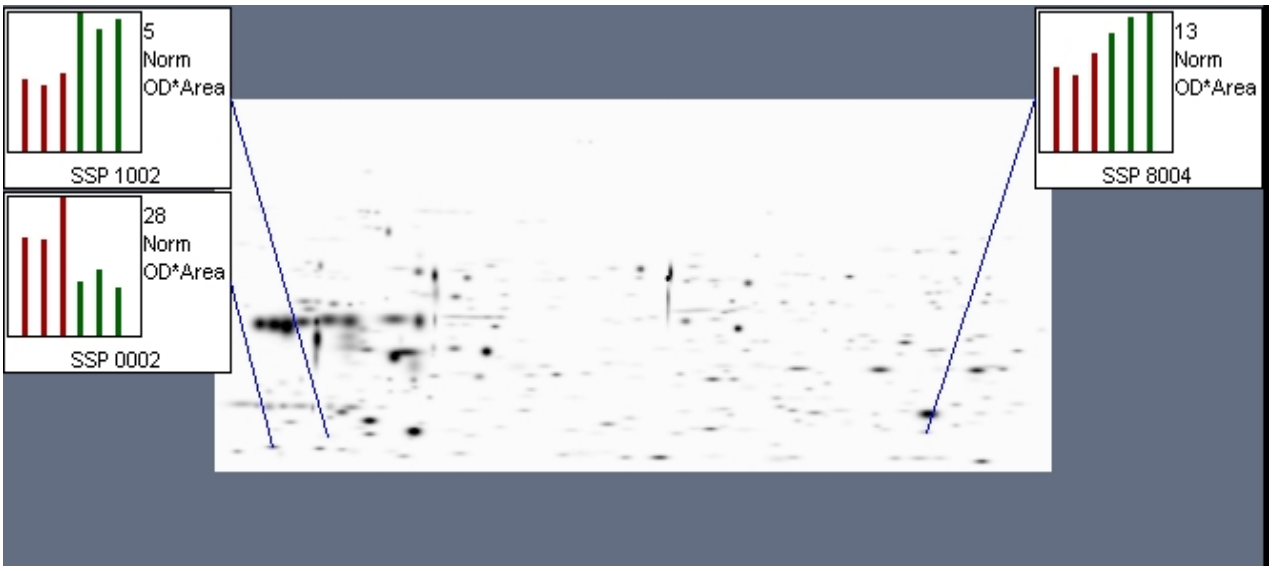


Figure 3a: Representative 2D SDS PAGE image of significantly differentially expressed proteins. SSP 1002: Hippocalcin; SSP 0002: Synaphin-1; SSP 8004: Neuronal protein NP25. Red histograms represent traumatized animals and green histograms controls, each sample run in triplicate.

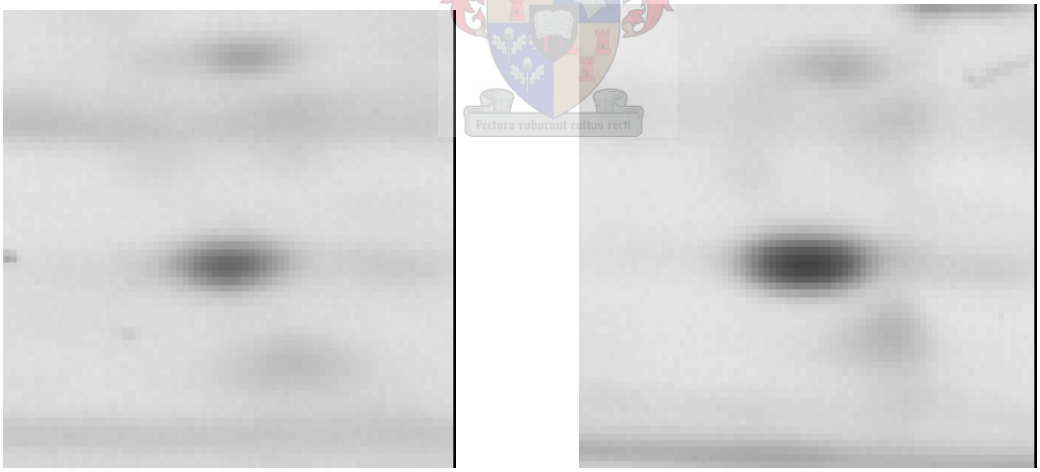


Figure 3b: Representative image of differential expression of Synaphin-1 (SSP 0002) between control 68 (left) and TDS 68 (right) animals. Synaphin shows a 2-fold increase in traumatized animals relative to control (see table 2).

Discussion

Clinical studies suggest that early life trauma may cause behavioural abnormalities later in life (Heim and Nemeroff, 2001; Kendler et al., 1992). However the underlying molecular mechanisms responsible for mediating the effects of the trauma, remains unclear. Using an animal model we are able to show that trauma during adolescence combined with adulthood re-stress affects a multitude of different cellular systems that include cytoskeletal, cell cycle, transcription, cell signaling, neuroplasticity and apoptotic proteins.

A number of indicators of cell dysfunction have been investigated in the present study. For instance the levels of S-100B, a member of the S100 family of EF-hand Ca^{2+}-binding proteins, are increased in the serum of both neurological patients that suffered an ischemic stroke, as well as psychiatric patients diagnosed with schizophrenia and major depression (Rothermundt et al., 2003). Interestingly, animal studies of prenatal stress have also showed increased serum levels of S-100B in adulthood (Barros et al., 2006). It has also been demonstrated that S-100B can be neuroprotective against NDMA-induced neurotoxicity in cultured rat hippocampal neurons (Kogel et al., 2004). This neuroprotection apparently resulted from the ability of S-100B to regulate Ca^{2+} homeostasis. Similar to prenatally stressed rats, animals subjected to trauma also displayed elevated S-100B levels compared to controls – a finding that we confirmed with Western Blot analysis. It is therefore likely that this increase in S-100B may result from raised Ca^{2+} concentrations in the neurons and/or astrocytes (Tramontina et al., 2006, Xiong et al., 2000).

Proline-rich tyrosine kinase 2 (Pyk2) is a non-receptor tyrosine kinase and is activated upon NMDA receptor stimulation (Alier and Morris, 2005). Pyk2 phosphorylation is increased after NMDA receptor stimulation and this increase is blocked by the PKC inhibitors, chelerythrine or staurosporine (Alier and Morris, 2005). In our experiments, trauma caused an increase in phosphorylated Pyk2, and this observation strengthens our hypothesis of stress-induced Ca^{2+} influx via NMDA receptors.

Hippocalcin is a Ca^{2+} binding protein in the hippocampus. Hippocalcin deficient mice show elevated levels of Caspase-12 (Korhonen et al., 2005), which is in agreement with the increase found in our traumatized animals. In addition high intracellular Ca^{2+} levels causes hippocalcin to translocate to the membrane (Oh et al., 2006), which may explain the decrease in the cytosolic extract sample of traumatized animals subjected to trauma.

Since Ca^{2+} is central to a number of intracellular signaling pathways, fluctuations in its concentration ought to translate into changes in the activity and expression of many other proteins. It was therefore not surprising to observe alterations in the levels of signaling proteins, especially those involved in stress-related responses. As such we report an increase in phospho- c-Jun N-terminal kinases (JNK's) expression in traumatized animals. JNK's are also called stress activated protein kinases and are part of the mitogen-activated (MAP) kinase family (Mielke and Herdegen, 2000). Both cytoplasmic and nuclear proteins can be phosphorylated by JNK's. These include neurofilament heavy chain (Giasson and Mushynski, 1997), Bcl-2 (Park et al., 1997), glucocorticoid receptors (Rogatsky et al., 1998) and c-Jun (Behrens et al., 1999). The increase in phospho-JNK, such as observed after trauma, once again reflect cellular stress. This is particularly plausible since we have previously shown a decrease in glucocorticoid receptors in traumatized animals (Uys et al., 2006b), possibly stemming from increased inhibition of its transcription by the JNK levels (Rogatsky et al., 1998). In addition we also found a decrease in MAP kinase kinase (MEK 1). This may point to an impairment in the cell's ability to transduce cell survival signals, since MEK inhibitors block NGF-induced neuroprotection after glutamate excitotoxicity (Singer et al., 1999).

Finally, the elevation in calcineurin further suggests that activity of Ca^{2+} mediated signaling has been enhanced in traumatised rats. This is in agreement with studies where animals subjected to chronic social stress, with impairment in long-term potentiation in the hippocampus, show higher basal levels of calcineurin (Gerges et al., 2004). Furthermore, calcineurin is also increased by S-100B in a Ca^{2+}-dependent manner (Leal et al., 2004).

Calcineurin (protein phosphatase 2B), is however also involved in neuronal plasticity (Groth et al., 2003) and so it was anticipated that changes in the expression of proteins participating in processes important for neural plasticity, would be present in rats subjected to trauma.

The protein, ARF nucleotide binding site opener (ARNO), which is a member of the ADP-ribosylation factor (ARF) family of small GTPases, is a regulator of dendritic branching in the hippocampus. It is present in both the embryonic and adult hippocampus and is upregulated during early dendritogenesis (Hernandez-Deviez et al., 2002). A down-regulation in ARNO levels is observed and this suggests impairment in dendritogenesis in traumatized animals. Similarly the Abl non-receptor tyrosine kinases family regulates cell morphogenesis and neuronal axonal development through interaction with the actin cytoskeleton (Gertler et al., 1995). Abl also modulates synaptic plasticity and neurotransmitter release in the hippocampus (Moresco et al., 2003). In addition, inhibition of Abl kinases decreases dendritic branching in hippocampal neurons (Jones et al., 2004). DRP-2 is important for guidance of axonal outgrowth and interacts with collapsin, which is responsible for dendritic guidance (Lubec et al., 1999). A decrease in DRP-2 could result in shortened dendrites and impairment in axonal innervation. A decrease in guanine deaminase activity, such as seen in trauma, is associated with impaired dentritic branching (Akum et al., 2004).
The decrease in guanine deaminase, DRP-2 and c-Abl caused by trauma is therefore aligned with the notion of reduced dendritogenesis and/or problematic short term synaptic plasticity.

Furthermore, under normal conditions Abl kinase activity is increased by integrin receptor engagement (Lewis et al., 1996). Another explanation for the reduced expression of c-Abl in the present study could be due to the increase in serine phospho-FAK (focal adhesion kinase) observed in traumatized rats. Focal adhesions link the extracellular matrix with the actin cytoskeleton and therefore control cell morphology (Yamakita et al., 1999). In addition, focal adhesions also play a role in integrin-mediated signal transduction. When FAK becomes phosphorylated on serine residues, there is a dephoshorylation on

tyrosine residues. This leads to the dissociation of the FAK/CAS/c-Src signaling complex and eventual integrin signaling arrest (Yamakita et al., 1999). Loss of integrin signaling has been associated with a specific type of apoptosis called anoikis (Frisch and Francis, 1994). Therefore in traumatized animals an increase in phospho-serine-FAK not only lead to disrupted neural plasticity but also to anoikis. Additional support for apoptotic pathways to be switched on following trauma, comes from data showing increased levels of caspases -9, -10 and -12. These cysteine proteases function as initiators of apoptosis and activate downstream executioners such as caspases -3, -6, and -7 (Riedl and Shi. 2004).

One of the major consequences of trauma is to disturb the integrity of the cytoskeleton of neurons. Tubulins are cytoskeletal proteins which aid in the growth and regulation of axons and dendrites. These extend from the cell body and give neuronal cells their asymmetrical morphologies. There are numerous α- and β-tubulin isoforms present which can also be post-translationally modified (Audebert et al., 1994). Polyglutamylation is the addition of 1-6 glutamyl units on the side chain of a glutamate residue near the carboxy terminus of either alpha or β-tubulin (Rudiger et al., 1992). This post-translational modified form of β-Tubulin is increased in neurons during differentiation (Audebert et al., 1994). Since trauma caused a decrease in polyglutamylated β-Tubulin, this may point to a decrease in neuronal differentiation.

Additional evidence relating to changes in important cytoskeletal components is seen in the increase in the concentration of desmin and cytokeratin in traumatized animals. Together with glial fibrillary acidic protein and vimentin, these proteins form intermediate neurofilaments in neurons. Changes in desmin and cytokeratin levels are therefore likely to indicate alterations in neuron structure, and this may contribute to cell nuclear fragmention and cell death (Yu et al., 1994). Large neuronal filaments (NFP) on the other hand are regulators of axonal transport. They can be divided into 200 kDa, 160 kDa and 68 kDa subunits respectively. Functional filaments are usually phosphorylated. Interestingly, Ca^{2+} overload induced a decrease in phospho-

NFP in the CA1 subfield and dentate gyrus of hippocampus of spontaneously hypertensive rats and these rats have increased susceptibility to ischemic damage (Sabbatini et al., 2001). It seems therefore that the reduced level of neurofilament 200 in traumatized rats is yet another indicator of neuronal stress.

Neuronal protein NP25 is a major interactor with actin and co-localizes with F-actin (Mori et al., 2004a). This interaction may be particularly important for reorganization during neuronal differentiation and normal neural development (Mori et al., 2004a). Furthermore, NP25 is increased during NGF differentiation of PC12 cells (Mori et al., 2004b). The decrease in NP25 in traumatized animals, together with the decrease in the growth factors, BDNF and NT-3 (Uys et al, 2006a), may point to an impairment in neuronal differentiation and actin stability of the cytoskeleton.

Syntaxin is a membrane protein, primarily expressed in neuronal tissue. It is predominantly responsible, together with synaptophysin and synapsin, for synaptic vesicle docking or fusion in the active zone of the presynaptic plasma membrane (Brose, 1993). Increases in the concentration of syntaxin have been observed in the frontal and temporal cortex of patients suffering from Alzheimer's disease (Mukaetova-Ladinska et al., 2000). Another protein associated with syntaxin, synaphin 1 (also called complexin II) (Ishizuka et al., 1999) was found to be increased in traumatized animals. Elevations in vesicular/docking proteins can therefore be associated with abnormal brain function.

The expression of MAP-1b, a structural protein, is significantly increased in animals subjected to trauma. Normally MAP-1b is expressed in high concentrations in the mossy fiber region of the hippocampus of the new born brain and then its level decreases with development (Schoenfeld et al., 1989). It has been shown that upregulation of MAP-1b occurs in the surrounding viable neurons of the infarct zone after cerebral ischemia (Popa-Wagner et al., 1999), suggesting a regenerative function of the protein. Interestingly the upregulation was greater in young rats than older animals (Popa-Wagner et

al., 1999). The increase in MAP-1b in our animals may therefore similarly reflect the activation of recovery mechanisms in the brain's attempts to combat the deleterious effects of the trauma.

The deleterious effects of trauma are not limited to signaling pathways and cytoskeletal proteins, but include regulators of the cell cycle. For instance Cdc25C and chk 1 expression are decreased and increased respectively. Cdc25C plays an important role in cell proliferation and cell cycle arrest. Inhibition of lung cancer cell growth by means of cell cycle arrest and apoptosis, was accompanied by reduced levels of Cdc25C and increased levels of c-Jun-N-terminal kinase (JNK) (Hsu et al., 2006). In addition, a decrease in Cdc25C and an increase in Chk1 were seen in malignant transformed Syrian hamster embryo cells by Malachite green (Ashra and Rao, 2005). This transformation of embryoninc cells was also associated with DNA damage and cycle cycle arrest. Alterations in the expression of other members of the cell cycle control machinery have also been obtained to support a claim of overall suppression of cell division. For example Cdk6, a D-cyclin associated kinase, play an important role in cell cycle control and cell division (Ericson et al., 2003). In addition, cdk7 with cyclin H and the assembly factor MAT1, together known as the CDK activating kinase (CAK) complex, are responsible for activating cdk6 (Lolli and Johnson, 2005). Both cdk6 (Costello et al., 1997) and cdk7 (Bicaku et al., 2005) have been found in human glioma cells and may play a role in transcription (Fisher, 2005). Finally, overexpression of cofilin, a regulator of cell motility via the actin cytoskeleton, leads to impaired cell motility and G1 phase cell cycle arrest (Lee et al., 2005). It may therefore be possible that the higher levels of cofilin together with the lower levels of cdk6 and cdk7 in traumatized animals are added markers of cell cycle arrest.

The above-mentioned proteins are not the only cell cycle proteins affected by trauma. Down-regulation of p16, a member of the INK4 family of proteins and an inhibitor of the cdk's (Sherr and Roberts, 1999), has been noted. While the INK4 family only binds to cdk4 and cdk6 and not to other cdk's or to D-type cyclins, the Kip family affects cyclin D-, E-, and A-dependent kinases (Sherr

and Roberts, 1999). More specifically, they are inhibitors of cyclin E- and A-dependent kinases and positive regulators of cyclin D-dependent kinases. $p57^{Kip2}$, which is required for normal development, and the overexpression thereof causes G1 phase arrest (Matsuoka et al., 1996), is upregulated after trauma. Similarly the expression of p34 (Cdc2), a cyclin B-dependent kinase is elevated in traumatized rats. Cdc2 catalyzes the phosphorylation of the protein BAD at serine 128. This results in BAD-induced apoptosis and inhibits the ability of growth factors to stop the apoptotic effect (Konishi et al., 2002). The increase in Cdc2 coupled with the upregulation in caspases 9, 10 and 12 (see earlier) and the decreased levels of the growth factors, BDNF and NT-3 in the ventral hippocampus (Uys et al., 2006a) strongly substantiates a condition of increased cellular stress leading to cell cycle arrest.

The effects of trauma have been so widespread that even nuclear structure and activity are altered. c-Myc is a transcription factor important in the regulation of both cell proliferation and cell death (Shervington et al., 2006). Even though c-Myc progresses the cell cycle from G1 phase to S phase, it also induces apoptosis when expressed under growth inhibitory conditions, such as lack of growth factors (Packham and Cleveland, 1995). As this is the case in traumatized animals (Uys et al., 2006a), the elevation in c-Myc levels further suggests a pro-apoptotic mode in which the cells may occur. Histones are proteins involved in the packaging of DNA into nucleosome units. Acetylation of the lysine residues on histones is associated with transcriptional activation and results in a remodeling of the nucleosome (Kornberg and Lorch, 1999). In addition, increased levels of histone acetylation in primary neurons, decreases the onset of apoptosis (Rouaux et al., 2003). The increase in acetylated histone 3 by trauma could therefore be a reaction by the cell to prevent and/or minimize apoptosis.

A number of energy metabolism ezymes are also dysregulated following trauma. These included ATPase, ADP-ribosylarginine hydrolase, vacuolar ATP synthase, pyruvate dehydrogenase and creatine kinase. This may reflect a general dysfunction of the cell and inability to supply adequate energy to function optimally.

In summary, subjection of adolescent rats to trauma results in extensive deleterious consequences. It remains to be investigated whether these effects occur within projection or interneurons. Our data suggests that the trauma caused prolonged NMDA receptor activation, leading to sustained (but not lethal) increases in intracellular Ca^{2+} concentrations. Because of its central role in cell function, a number of physiological systems are disturbed by the continuous elevation. Subsequently signaling pathways are dysregulated, the overall structure of the cell is disrupted, and apoptotic mechanisms are initiated. The fact that the animals are able to survive suggests that compensatory mechanisms which counter this death penalty are still in place and therefore prevent large scale cell death in the brain. These effects may occur in neurons and/or glial cells. However, the balance between these two opposing systems is tilted towards aberrant brain function as traumatized rats display abnormal behaviour (Uys et al., 2006a).

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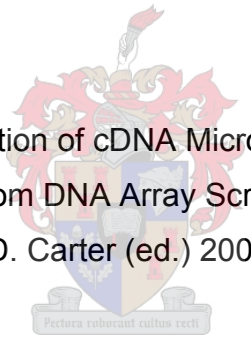

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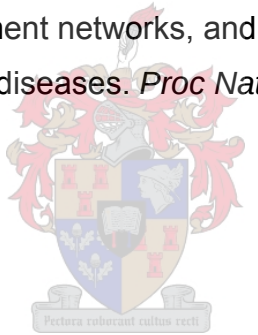


Chapter 9
General Conlusion

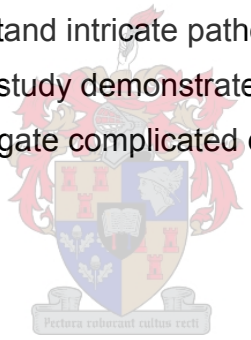
This study focused on the effects of early life trauma in adulthood. This was explored on a behavioural, neuroendocrinological and proteome level.

Animals subjected to adolescent trauma showed an increase in basal corticosterone levels and a decrease in glucocorticoid receptors in the dentate gyrus 24 hours after adulthood re-stress (PND 61) (Chapter 3). These effects were reversed by pre-treatment with the SSRI, escitalopram. In addition, there were no changes in NGF, BDNF or NT-3 at this timepoint (Chapter 4). Decreases in BDNF in the dorsal and in NT-3 in the dorsal and ventral hippocampus were evident 8 days post re-stress (PND 68), with an increase in basal corticosterone levels and behavioural hyperarousal (Chapter 5). The changes in neurotrophins were not accompanied by changes in plasma BDNF or NT-3 levels or PI-3 kinase, PKB, PTEN or phospho-Forkhead expression in the dorsal and ventral hippocampus (Chapter 6).
A number of cell cycle-, signaling-, plasticity- and apoptotic proteins were up- or down regulated in the ventral hippocampus on PND 68 (Chapter 8).

We propose that adolescent trauma causes an increase in corticosterone levels which leads to down regulation of GC receptors, possible through JNK mediated inhibition of GC receptor transcription. The trauma-induced down regulation of GC receptors causes basal corticosterone levels to stay elevated by a decrease in negative feedback inhibition. It has been proposed that an increase in glucorticoids may damage neurons through a NMDA receptor dependent mechanism and increase stress-induced neuronal susceptibility to subsequent insults (Armanini et al., 1990). Indeed, high levels of corticosterone in rat hippocampal neurons, caused a rapid and prolonged elevation of Ca^{2+} through NMDA receptors, which resulted in Ca^{2+} induced

neurotoxicity (Takahashi et al., 2002). The decrease in BDNF and NT-3 on PND 68 may contribute the cell's susceptibility to subsequent stressors. These effects, together with the loss of integrin signaling and an increase in caspase initiator proteins, indicate that the brain of traumatized rats are in a state of pro-apoptosis, specifically anoikis. In addition a number of cell cycle arrest proteins are upregulated. Cell cycle arrest during G1/S phase provides resistance to anoikis (Collins et al., 2005). The parallel processes of the cell to avoid anoikis on one hand and inducing a state of cell cycle arrest on the other, is probably a biological coping mechanism to avoid transcription of sub-standard cellular features.

This study showed that adolescent trauma caused a number of changes on behavioural and cellular level. In addition, it highlights the complexity of behavioural-cellular interactions and underlies the necessity to study multi-parameters if one is to understand intricate pathologies such as mood and anxiety disorders. Finally, the study demonstrated that proteomic technologies provide us with tools to investigate complicated disease states in a more holistic manner.



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